

**SYNTHESIS OF UV-ABSORBING CARRIER AMPHOLYTES FOR
CHARACTERIZATION OF ISOELECTRIC MEMBRANES**

A Thesis

by

ANN HWANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Chemistry

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Approved by:

Chair of Committee,
Members,

Head of Department,

Gyula Vigh
David H. Russell
James C. Hu
Emile A. Schweikert

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ABSTRACT

Synthesis of UV-absorbing Carrier Ampholytes for Characterization of Isoelectric Membranes. (August 2005)

Ann Hwang, B.S., University of Illinois at Urbana – Champaign

Chair of Advisory Committee: Dr. Gyula Vigh

Isoelectric focusing is one of the most important techniques in protein separations. Preparative-scale isoelectric separations often use buffering membranes (isoelectric membranes), but there are no good known methods for the characterization of their pI values. Therefore, UV-absorbing carrier ampholyte mixtures (UVCAs) have been synthesized, analytically characterized, and utilized for the characterization of the pI value of a buffering membrane.

To synthesize the UVCAs, addition of a UV-absorbing electrophile, 3-phenoxypropyl bromide (PhOPrBr), to a pentaethylenhexamine (PEHA) carrier ampholyte backbone, resulted in an intermediate that was subsequently reacted with increasing amounts of acrylic acid (up to 8 equiv) and itaconic acid (up to 2 equiv) via Michael's addition. The intermediates and final products were characterized by ^1H -NMR and full-column imaging capillary isoelectric focusing techniques.

An optimal blended mixture of selected UVCAs was first desalted and purified by isoelectric trapping and its composition verified by full-column imaging isoelectric focusing. The mixture of UVCAs possessed a broad pI distribution from approximately pH 3 – 10. By isoelectric trapping, the mixture was separated into two subfractions with a polyacrylamide-based isoelectric membrane of known pI as the separation membrane and poly(vinyl) alcohol-based buffering membranes as the restriction membranes. The pI of the most basic UV-active carrier ampholyte in the anodic fraction was determined to be 4.4 and the pI of the most acidic UV-active carrier ampholyte in the cathodic fraction was determined to be 4.4, confirming that the pH of the polyacrylamide-based isoelectric membrane was pH 4.4.

To my soul mate, my rock, my h.g. – for his unconditional love and support.

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CHAPTER I

INTRODUCTION

1.1 Isoelectric Focusing

Isoelectric focusing (IEF) has rapidly gained widespread acceptance across chemical and biological fields in the last 35 years. IEF is a high resolution analytical and preparative separation technique [1]. Traditionally, IEF is performed in polyacrylamide gel slabs (gel IEF). On the analytical scale, this technique tends to be slow, labor-intensive, and not quantitative. Therefore, IEF in capillaries was first introduced by Hjertén and Zhu in 1985 [2]. They demonstrated the advantages of capillary IEF (CIEF) over gel IEF on the analytical scale by eliminating the drawbacks of gel IEF.

In IEF, when an ampholytic species is introduced into a prefocused pH gradient, it migrates to a place where its isoelectric point (pI) is equal to the pH of the environment. At this point, the net electrical charge of the species will be zero, thus it ceases to migrate further. If the ampholytic molecule migrates or diffuses away from its focused position, it develops a net charge and moves back to its focused position. Thus, the ampholytic molecule will reach an equilibrium position where it concentrates into a narrow band [3, 4].

This thesis follows the style and format of *Electrophoresis*.

The key to this steady-state separation technique is the formation of a stable pH gradient under the influence of an electric field. A mixture of ampholytic molecules, called carrier ampholytes, is used to create a monotonous pH gradient from the anode to the cathode [3]. Carrier ampholytes are polyamino – polycarboxylic acids that electrophoretically establish a stable pH gradient, in which the pH progressively increases from anode to cathode in a suitable anticonvective medium. These relatively small ampholytic molecules possess good buffering capacity and conductivity.

In CIEF, after the focusing process, the focused analyte zones are generally mobilized for single-point detection. The capillary format offers a range of detection methods. Among the most common are ultraviolet absorption (UV) and laser-induced fluorescence (LIF) detection. However, detection at a fixed point which requires a mobilization step, poses some problems, including: disturbance of the even pH gradient and longer analysis time, which leads to decreased resolution and reproducibility [5]. A full-column imaging detection system eliminates these problems [6].

Three types of imaging detection systems were developed to overcome the problems of single-point detection: moving capillary, spatial scanning, and whole-column imaging [6]. Moving the separation capillary through the detection window also has problems: the dynamic noise is relatively high and zone

distortions occur due to movement of the capillary [7]. Spatial-scanning allows for monitoring the dynamic focusing process, but is limited by the slow scan speed (more than 15s over an 8-cm capillary) [8]. Whole-column imaging detection (iCIEF) offers the best solution to the problems of single-point detection. Proteins [6, 9 – 12] and peptides [6, 10] have been analyzed in whole-column imaging mode. The commercially available iCIEF instrument set-up is shown in Figure 1 [6].

1.2 Isoelectric Trapping Separations

Although highly successful and practical, the IEF technique poses some problems. First, the pH gradient formed by carrier ampholytes is not temporally stable because the lowest and highest pI carrier ampholytes are lost to the electrolyte compartments during electrophoresis. As the separation occurs, the most acidic and most basic ampholytes in the pH gradient migrate out of the separation compartment, into the anode and cathode compartment, respectively, resulting in a flat plateau in the middle of the gradient. This is especially disconcerting for long separations [13]. Second, when the analytes are present in isoelectric state, precipitation can occur because the analytes aggregate. Additionally, in preparative applications, separation of the carrier ampholytes from the separated target analytes is very difficult. Thus, isoelectric trapping (IET) was developed to eliminate the short-comings of preparative-scale IEF.

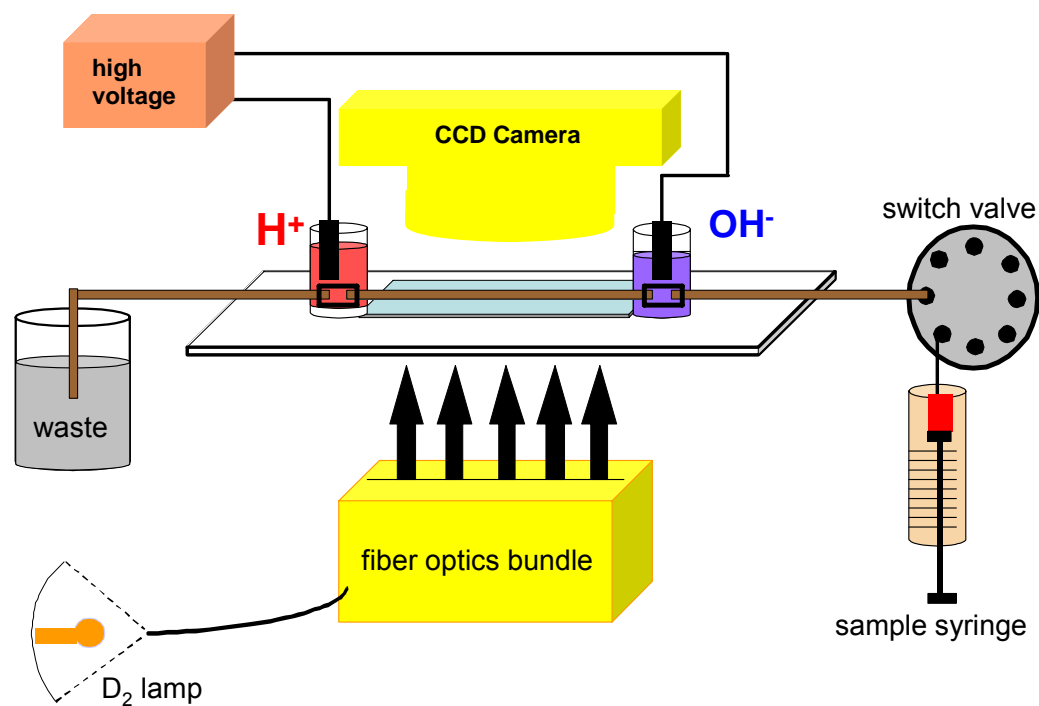


Figure 1. The commercially available iCIEF instrument set-up

Multi-compartment electrolyzers (MCEs) were first produced for preparative isoelectric trapping separation of proteins by Martin et al. [14]. In isoelectric trapping (IET), an analyte of interest is trapped between two buffering membranes whose pH is above and below the pI value of the analyte. The membranes prevent convective mixing between the adjacent compartments of the MCE and allow the passage of ions.

MCEs have evolved into very successful, rugged, and useful instruments. One of them, the modified Gradiflow BF200IET unit is shown in Figure 2 [15]. With this IET unit, the anolyte and catholyte are simultaneously recirculated and cooled to remove Joule heat [16]. Additionally, the sample streams can be either re-circulated or sent through in pass-by-pass mode. In pass-by-pass mode, the entire sample volume is passed through the separation cartridge and is collected before it is passed through the separation cartridge again. Pass-by-pass separations are repeated until the target analytes migrate to their desired chamber, which marks the end of the separation.

1.3 Buffering Membranes (Isoelectric Membranes)

Currently, in IET separations, buffering (isoelectric) membranes are made by copolymerizing acrylamide with N, N'-methylenebisacrylamide, and an appropriate acrylamido weak or strong acid and an appropriate acrylamido weak

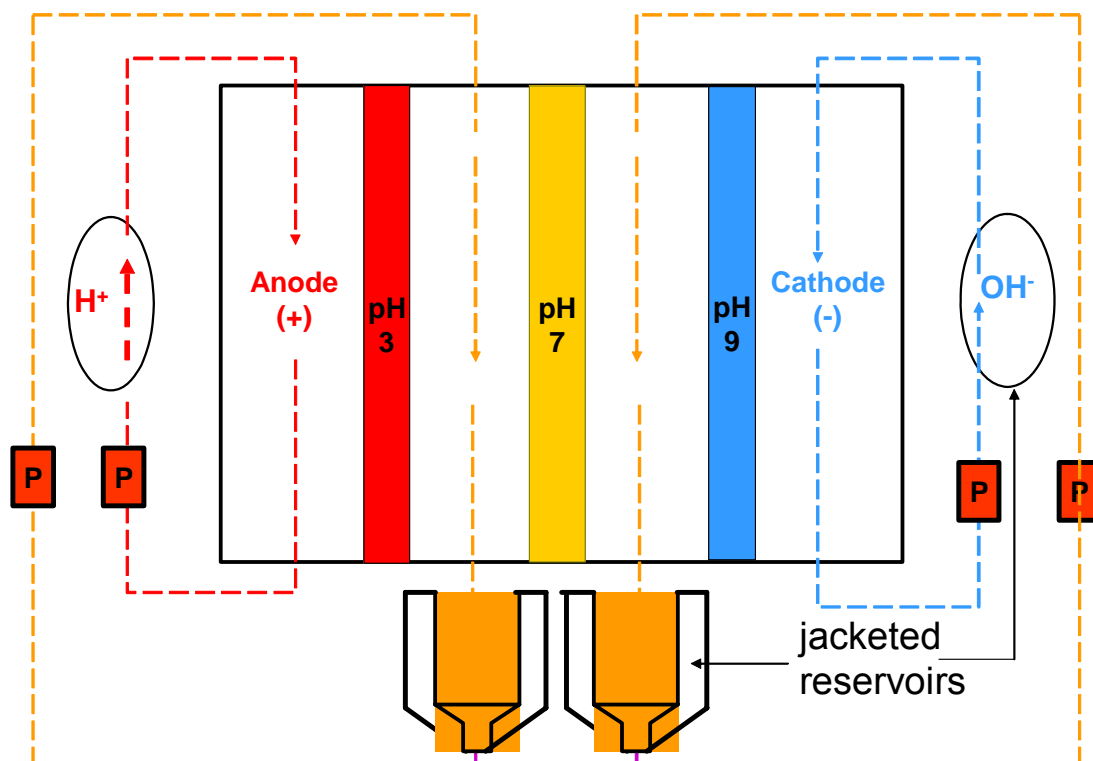


Figure 2. The modified Gradiflow BF200IET unit

or strong base. By systematically changing the type and amounts of each, the pH of the membrane can be changed over the pH range 3 – 10. These polyacrylamide-based buffering membranes have been supported on glass fiber fillers [17] and woven poly(ethylene terephthalate) substrates [15].

Although proven to be highly useful and highly successful, polyacrylamide-based membranes are hydrolytically unstable at the extreme pH regions, including pH < 3 and pH > 9. Recently, however, hydrolytically-stable low- and high-pH buffering membranes have been synthesized for IET separations [18, 19].

These membranes are made by crosslinking poly(vinyl alcohol) with glycerol-1, 3- diglycidyl ether and attaching an ampholytic species that is selected according to the membrane pH needed. The limitation of these membranes, however, is that their operational pH value is reported only as a range and not as a single value as for the polyacrylamide-based buffering membranes because there has not been a good experimental method for the determination of the pH of the membrane.

1.4 Synthesis of Carrier Ampholytes

Progress towards the synthesis of new carrier ampholytes has been slow for the last few decades. Carrier ampholytes are essential for classical isoelectric

focusing (IEF) techniques, for example, capillary IEF. The known syntheses of carrier ampholytes have been limited to UV-transparent carrier ampholytes.

Svensson laid the theoretical foundation [20] for the synthesis of carrier ampholytes and Vesterberg was the first to experimentally prove Svensson's predictions [3]. Svensson laid the criteria for carrier ampholytes, in that they must have good buffering capacity and good conductivity at their pI values, and form a stable pH gradient under the influence of an electric field [20]. Carrier ampholytes, therefore, must have closely spaced pKa values that straddle their pI value. Vesterberg realized that to create a pKa scaffold required the use of a molecule with acidic and basic groups, which possess closely spaced pKa values [21, 22]. This was achieved by using low molecular weight oligoamines such as ethylenediamine, diethylenetriamine, triethylenetetramine, and tetraethylenepentamine [22]. Commercially available Ampholines [3, 23] are based on Vesterberg's synthesis in which acrylic acid, methacrylic acid, maleic acid, crotonic acid, and itaconic acid are added to oligoethylenimine [22]. These reactions proceed via an anti-Markovnikov Michael addition [3, 22 – 30]. The UV-transparent carrier ampholytes produced can form a stable pH gradient upon electrolysis and offer good buffering capacity and conductivity [21, 22].

One drawback of the Vesterberg and Svensson synthesis was the poor performance of the carrier ampholytes in the 4.5 to 6.6 pH range. Four types of

carrier ampholytes were made from hexamethylenetetramine (HMTA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA) and pentaethylenehexamine (PEHA) by adding acrylic acid to them. Then, by mixing the four types of carrier ampholytes, a much smoother pH gradient could be produced over the 3 to 9.5 pH range [24]. Further investigation revealed that commercial carrier ampholytes, which are polyamines coupled to acrylic acid [22], have a conductivity minimum in the 5.5 to 6.5 pH range. To alleviate this problem, itaconic acid was used instead of acrylic acid because its pK_a values ($pK_{a1}=3.85$; $pK_{a2}=5.45$) are nearer to that range [25]. PEHA carrier ampholytes have also been synthesized for use in IEF separations of immunoglobulines in agarose gels [26].

To extend the pH range, Servalytes were synthesized using the product of propyldiamine with aziridine as the polyamino backbone, shown in Figure 3.

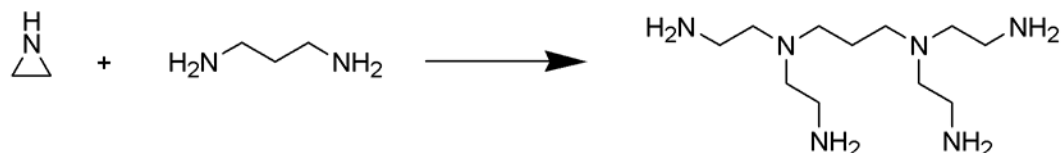


Figure 3. Synthesis scheme of the polyamino backbone for Servalytes

The product is distilled to exclude compounds with molecular weights greater than 400. Due to the presence of tertiary amine groups in the product, the pK_a 's

are higher and therefore, there is a wider pH range. Rather than using acrylic acid like Vesterberg, Serva used propane sultone and sodium vinylsulfonate for the sulfonic acid group, and the sodium salt of chloromethyl phosphate for the phosphinic acid group, yielding the product shown in Figure 4.

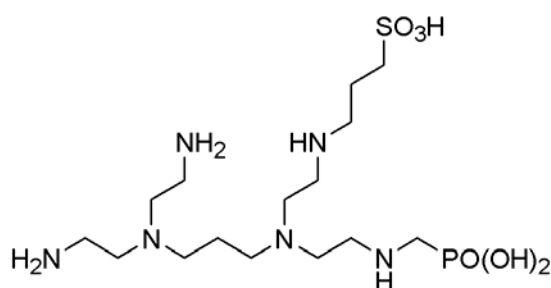


Figure 4. Servalyte product

The incorporation of carboxyl, sulphate, and phosphate groups to the polyamino backbone widened the pH range from 2 to 11 [3].

Contrary to other carrier ampholytes, Pharmalytes are first made in five narrow pH ranges and then blended together to produce a pH range of 3 to 10. Pharmacia further increased the structural diversity of the ampholytes by using D,L-epichlorohydrin, D,L-amines, and D,L-amino acids, including the diastereomers of the ampholytes [3], resulting in a significant increase in the number of components with different pI values.

Righetti and Hjertén transitioned from using carrier ampholytes for analytical scale to preparative scale IEF separations by synthesizing high molecular weight carrier ampholytes for the IEF separation of peptides [10]. This was accomplished by reacting high molecular weight PEI (M_{avg} 40,000 – 60,000) with increasing amounts of acrylic acid [30].

Rodkey and DeShong further increased the heterogeneity of Versterberg's original carrier ampholytes by crosslinking the polyamines or peptides with D,L -, meso and racemic forms of tartaric acid esters, malonic acid esters, and other dicarboxylic acid esters. The crosslinked polyamines and or peptides were reacted with one or a mixture of the following α , β -unsaturated carboxylic acids: methacrylic acid, methylene malonic acid, ethylene malonic acid, crotonic acid, maleic acid, fumaric acid, and itaconic acid. This produced a large number of heterogeneously crosslinked polymers whose pI values cover a broad range and can be fractionated to obtain narrow pI cuts [27 – 29].

The amines used in their synthesis were diethylenetriamine (DETA), triethylenetetramine (TETA), TEPA, and PEHA. The peptides used contain at least two amino groups and a chain of 3-10 amino acids. The amino acids used were D, L-lysine, arginine, histidine, and mixtures of them. The lower molecular weight peptides are usually separated from the larger molecular weight proteins after IEF [27 – 29].

Recently, a new isoelectric buffer for capillary electrophoresis was reported. This ampholytic polymer, *N*-carboxymethylated PEI was synthesized for the near neutral pH range of 6.8 by reacting PEI with sodium chloroacetate in an aqueous environment, with a 1:2 mole ratio of acid:base [31].

1.5 Alkylation of PEHA

Different synthetic approaches have been used to obtain a secondary amine from a primary amine, including; reductive alkylation and protecting group chemistry [32, 33]. Additionally, direct N-alkylation of primary amines has been cited numerous times in the literature [34 – 37]. Despite its success, direct N-alkylation techniques have been inefficient due to overalkylations. Therefore, to solve this problem, a method to prepare secondary amines by selectively alkylating a primary amine with an alkyl bromide was developed using cesium hydroxide [38].

Another strategy for the N-alkylation of primary amines using alkyl bromide was developed using Me₂SO as a solvent and K₂CO₃ as a base. This design selectively produced either mono or dialkyl amines. A 1:1 molar ratio of amine:alkyl bromide was used. Shorter alkyl chains with 2 – 3 carbon atoms required 6 – 8 hours of reaction time and the products were obtained in high yields (> 92%) [39].

CHAPTER II

OBJECTIVES AND RATIONALE

2.1 Determination of the pH of Buffering Membranes

Preparative-scale IEF often uses buffering membranes, but there are no good known methods for the characterization of their operational pH values. If a mixture of UV-active carrier ampholytes with a broad pI distribution were available, the mixtures could be separated with the buffering membrane in question into two subfractions. By determining the pI of the most basic UV active carrier ampholyte in the anodic fraction and the pI of the most acidic UV active carrier ampholyte in the cathodic fraction, the operational pH value of the buffering membrane can be determined.

2.2 Carrier Ampholyte Backbone

The most promising candidate for a carrier ampholyte backbone will not only possess the qualities of Vesterberg-type carrier ampholytes, but more importantly, possess a range of pKa's on a single chain. The backbone used in this study was pentaethylenehexamine (PEHA). According to the pH titration curve of PEHA, the pKa's are so closely spaced that it is very difficult to discern

the actual pKa values [23]. However, the pKa's of PEHA should follow the same pattern as shown for the shorter polyamines in Table 1 by Vesterberg [21].

Table 1. pKa's of polyamines (Vesterberg)

Amine	pKa₁	pKa₂	pKa₃	pKa₄	pKa₅
Ethylenediamine	10.1	7.0			
Diethylenetriamine	9.9	9.1	4.3		
Triethylenetetramine	9.9	9.2	6.7	3.3	
Tetraethylenepentamine	9.9	9.1	7.9	4.3	2.7

2.3 UV-absorbing PEHA-based Carrier Ampholytes

UV absorbing carrier ampholytes (UVCAs) are essential for the characterization of the operational pH value of buffering membranes. Therefore, a UV-absorbing functional group needs to be attached to the backbone of the carrier ampholytes. There are a few considerations for choosing the appropriate candidate. Firstly, the instrument used to determine the pI values is the iCE280 instrument from Convergent Biosciences, which only operates at a wavelength of 280 nm. Secondly, carrier ampholytes generally have very low light absorption above 260 nm in a 1% solution [21]. Therefore, a chromophore with a high molar absorptivity at 280 nm is needed.

Some potential chromophore candidates that meet this requirement are nitrobenzyl, naphthyl, and dansyl groups. However, nitrobenzyl groups are not good candidates for two reasons. It introduces another electroactive functionality ($-\text{NO}_2$) which, once altered in an electrode reaction, will cause a change in the overall pI of the carrier ampholytes. Naphthyl compounds are also poor candidates because they are too hydrophobic due to the bicyclic aromatic structure. Similarly, dansyl compounds are also too hydrophobic due to the naphthyl ring, but will also, upon reaction, lead to a change in the overall pI value of the carrier ampholytes. Thus, alkoxybenzyl chromophores are logical candidates.

The next criterion for the synthesis of UVCA is choosing a candidate with good reactivity, such as alkyl halides. Among the halides; bromo and iodo alkanes are the two best. The iodo group is the best leaving group, however, only 3-phenoxypropyl bromide is commercially available. If this reagent is used, then a catalytic amount of potassium iodide can be added to the reaction mixture. Therefore, a nucleophilic bimolecular substitution ($\text{S}_\text{N}2$) reaction will occur between PEHA (carrier ampholyte backbone) and 3-phenoxypropyl bromide producing a UV-absorbing PEHA. Subsequently, the Michael addition will be performed in an anti-Markovnikov fashion with acrylic and/or itaconic acid to finally produce the UV-absorbing carrier ampholytes.

CHAPTER III

SYNTHESIS AND CHARACTERIZATION

In this work, UV-absorbing, PEHA-based carrier ampholytes have been synthesized and analytically characterized on small and large scales.

3.1 Chemicals

Pentaethylenhexamine (PEHA), 3-phenoxypropyl bromide (PhOPrBr), acrylic acid, itaconic acid, potassium iodide, sodium hydroxide, methanesulfonic acid (MSA), phosphoric acid, and methylcellulose (MC, average relative molecular mass 65 000), sodium sulfate, arginine (ARG, $pI = 10.7$), and iminodiacetic acid (IDA, $pI = 2.2$), were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). The pI markers, dansyl-DL-aspartic acid (DNS Asp, $pI = 3.2$), dansyl phenylalanine (DNS Phe, $pI = 3.52$), m-aminobenzoic acid (MABA, $pI = 3.9$), terbutaline (TER, $pI = 9.6$), dopamine (DOPA, $pI = 9.75$), and tyramine (TYRA, $pI = 10.0$), were obtained from Sigma (St. Louis, MO, USA). The UV-transparent carrier ampholytes, Pharmalytes pH 3 – 10, were from Amersham Biosciences AB (Uppsala, Sweden) and the NMR solvents, deuterated methanol (CD_3OD , 99.9%) and deuterium oxide (D_2O , 99.9%), were from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All solutions for the Michael additions were freshly prepared using deionized water from a Milli-Q unit

(Millipore, Milford, MA, USA). All of the solutions and pl markers for the full-column imaging iCE280 instrument were filtered through a 0.45 μm PVDF membrane filter (Millipore, Bedford, MA, USA).

3.2 General Methods

The reaction between PEHA and PhOPrBr was monitored by ^1H NMR using a UnityPlus 500 spectrometer with a quad nucleus (^1H / ^{19}F / ^{31}P / ^{13}C) probe utilizing the Vnmrx 5.3b software. Similarly, the Michael additions between the UV-absorbing PEHA and acrylic acid and/or itaconic acid were also monitored by ^1H NMR using the same instrument, probe, and software.

The pl range of the UV-absorbing PEHA carrier ampholytes was determined by using the full-column imaging iCE280 instrument from Convergent Biosciences, Ltd. (Toronto, CANADA). The separation cartridge, also from Convergent Biosciences, contained anolyte and catholyte reservoirs separated by a 5 cm long, 100 μm I.D. fused-silica capillary that was internally coated with a fluorocarbon polymer. The exterior coating of the capillary was removed. The anolyte and catholyte was 80 mM phosphoric acid and 100 mM sodium hydroxide, respectively. To suppress the electroosmotic flow, the solutions contained 0.1% MC [10, 18]. The iCE280 instrument was linked to an Alcott 718AL autosampler from Alcott (Norcross, GA, USA) with a 96-well microtiter

plate. The imaging CIEF (ICIEF) separations were completed in 10 min at 3 kV with a 140 second transfer time and 0.5 min desalting time at 500 V. The electropherograms were imaged at 280 nm. The carrier ampholytes used were 4% Pharmalytes pH 3-10 for each of the analyte samples. Each sample was also loaded with different pI markers depending on the sample. Then, the total volume in each well of the microtiter plate was brought to 200 μ L with 0.35% MC. Lastly, 35 μ L of the mixture was loaded onto the iCE280.

The IET separations were completed with a BF200IET unit from Life Therapeutics (French's Forest, NSW, Australia). Also from Life Therapeutics were the Immobiline – polyacrylamide buffering membranes containing a poly(ethylene terephthalate) substrate from Mitsui & Co. (Melbourne, Australia). In addition, poly(vinyl alcohol)-based buffering membranes [18, 19] were also used. The entire system, also called a Twin Flow system, was continuously cooled with ice-water to remove the Joule heat generated. The anolyte, 30 mM methanesulfonic acid (MSA), and catholyte, 150 mM sodium hydroxide, were continuously recirculated at a flow rate of 2 L/min. A DC power supply, PS1000 from Thermo Savant (Holbrook, NY, USA) was used. Some experiments were performed in “pass-by-pass” operation mode [16], while others were performed such that the sample streams were continuously recirculated through the separation head. In the “pass-by-pass” mode, 0.5 mL aliquots were taken to

measure the pH and conductivities. From IQ Inc., a solid-state microelectrode, pH 16-SS, model IQ240, pH meter was used (San Diego, CA, USA). From Microelectrodes, a model MI-905 conductivity microprobe was used (Bedford, NH, USA) along with a model 145A+ conductivity meter from Thermo-Orion (Beverly, MA, USA). Additionally, the current, potential, and power were monitored throughout the course of the IET separations.

3.3 Synthesis of UV-absorbing PEHA

To make a UV-absorbing carrier ampholyte, PEHA was first converted into a UV-active material by alkylating it with 3-phenoxypropyl bromide. To a 1-L, 3-neck round-bottom flask, PEHA was added and stirred using a 3.5 cm football-shaped stir bar. A Leibig condenser was attached to the middle neck with a mercury thermometer in another neck. A catalytic amount of KI, 0.03% (2.157 g, 0.013 mol), was added to the reaction flask. While stirring the PEHA / KI mixture, the reaction flask was heated to approximately 30 °C before PhOPrBr was added dropwise in 5 hours. The PEHA to PhOPrBr molar ratio was 1:1. The reaction mixture was then heated to 70 °C. The temperature of the reaction was controlled by controlling the drop rate of PhOPrBr. During the entire process, N₂ gas was purged through the system. The resulting product was a clear, orange viscous material. The reaction scheme is shown in Figure 5. ¹H NMR was used to confirm that the alkylation reaction shown in Figure 6 took

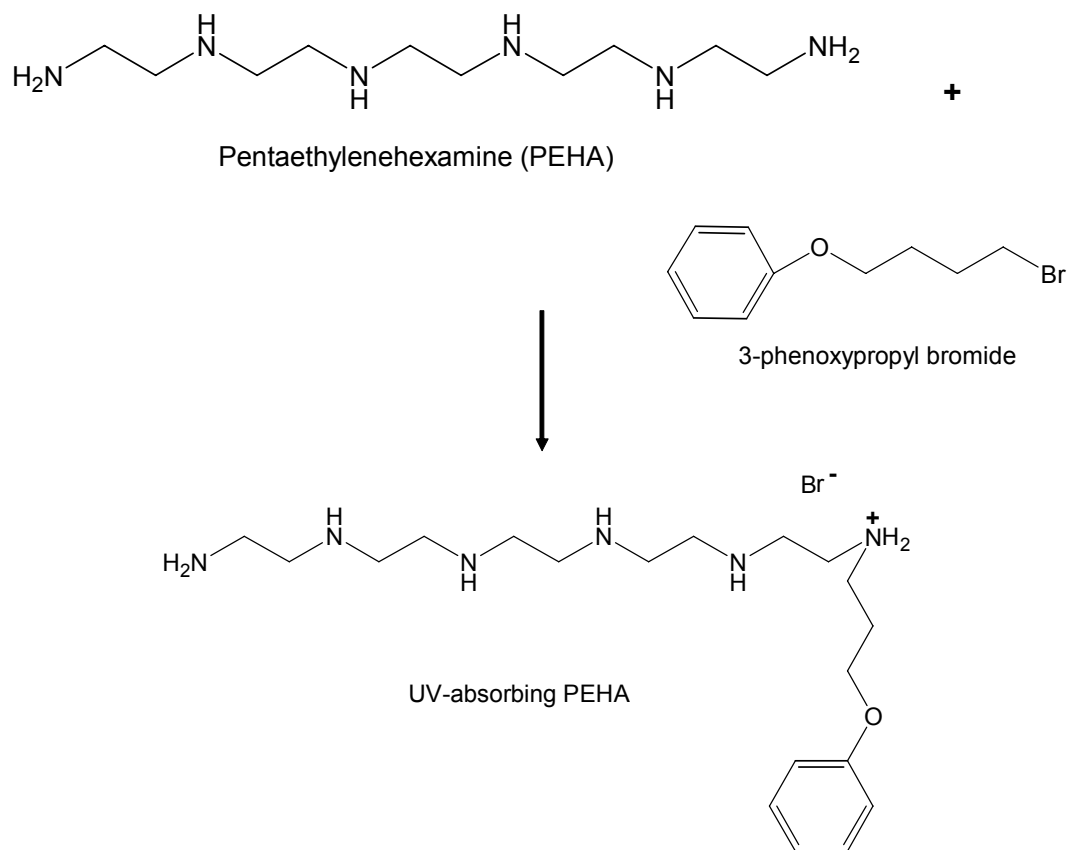


Figure 5. Alkylation reaction

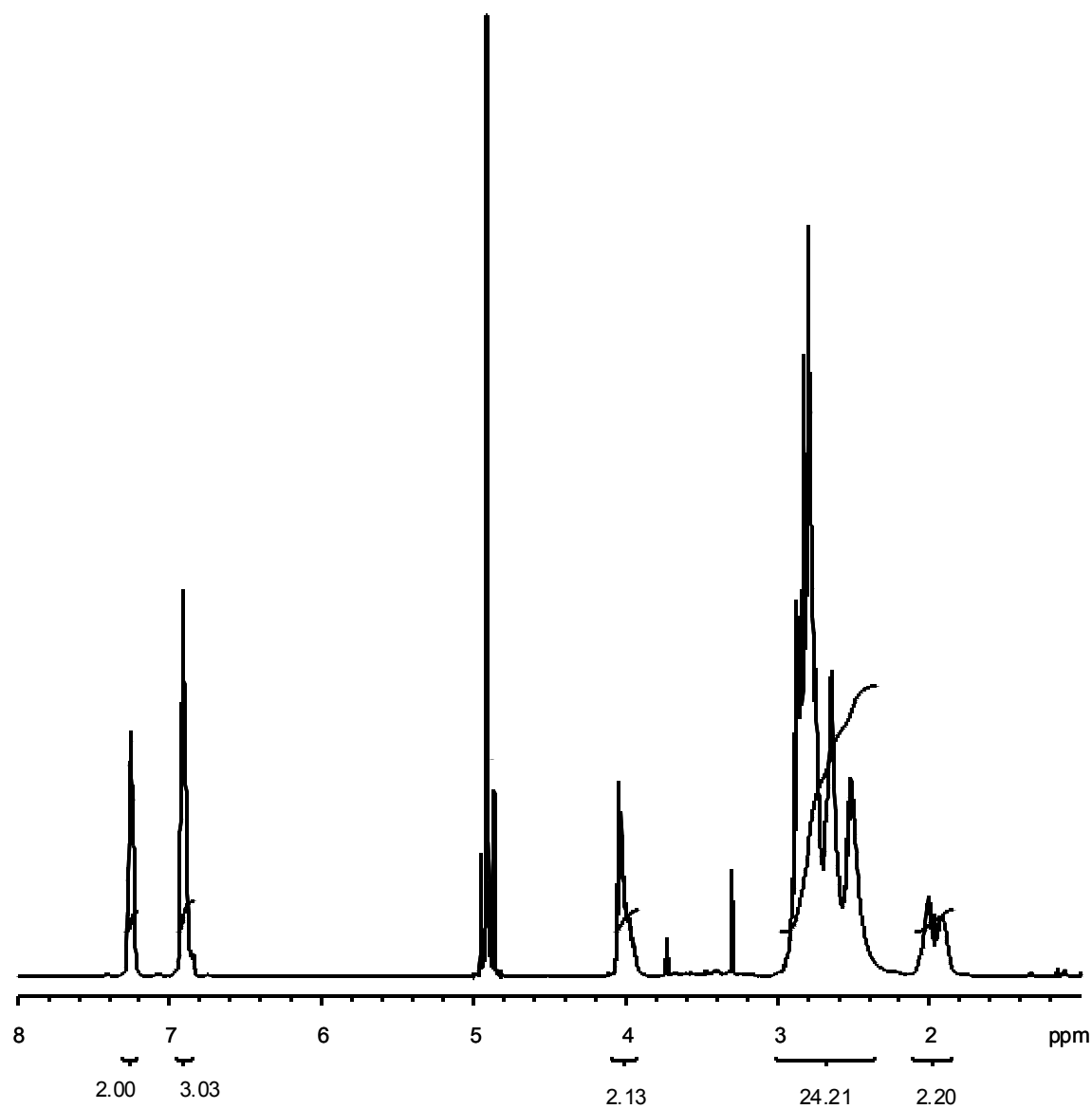


Figure 6. ^1H NMR to confirm completion of the alkylation reaction

place. The ^1H NMR spectra of the starting materials, PEHA and PhOPrBr, are shown in Figures 7 and 8, respectively.

From the ^1H NMR, it is evident that the reaction was successful for several reasons. Firstly, the characteristic phenoxy peaks are observed at 6.9 and 7.3 ppm. However, due to the alkylation, these signature peaks from the ring have shifted slightly with respect to the original starting material. The peak shapes are slightly different and show a finer splitting pattern. Secondly, the ^1H NMR of PEHA shows resonances of the active protons on the backbone at 2.45 – 2.85 ppm. Once the substitution reaction occurred, the signals shifted upfield to the 1.9 – 3.0 ppm range. This change indicates that incorporation took place because the proton environments are now different. Thirdly, there is a new signal at ~4 ppm indicating another change in the environment of one of the amine protons.

3.4 Synthesis of UV-absorbing PEHA-based Carrier Ampholytes

By Michael's addition, following the anti-Markovnikov rule, UV-absorbing PEHA carrier ampholytes have been synthesized using acrylic acid and/or itaconic acid on both small and large scales and analytically characterized.

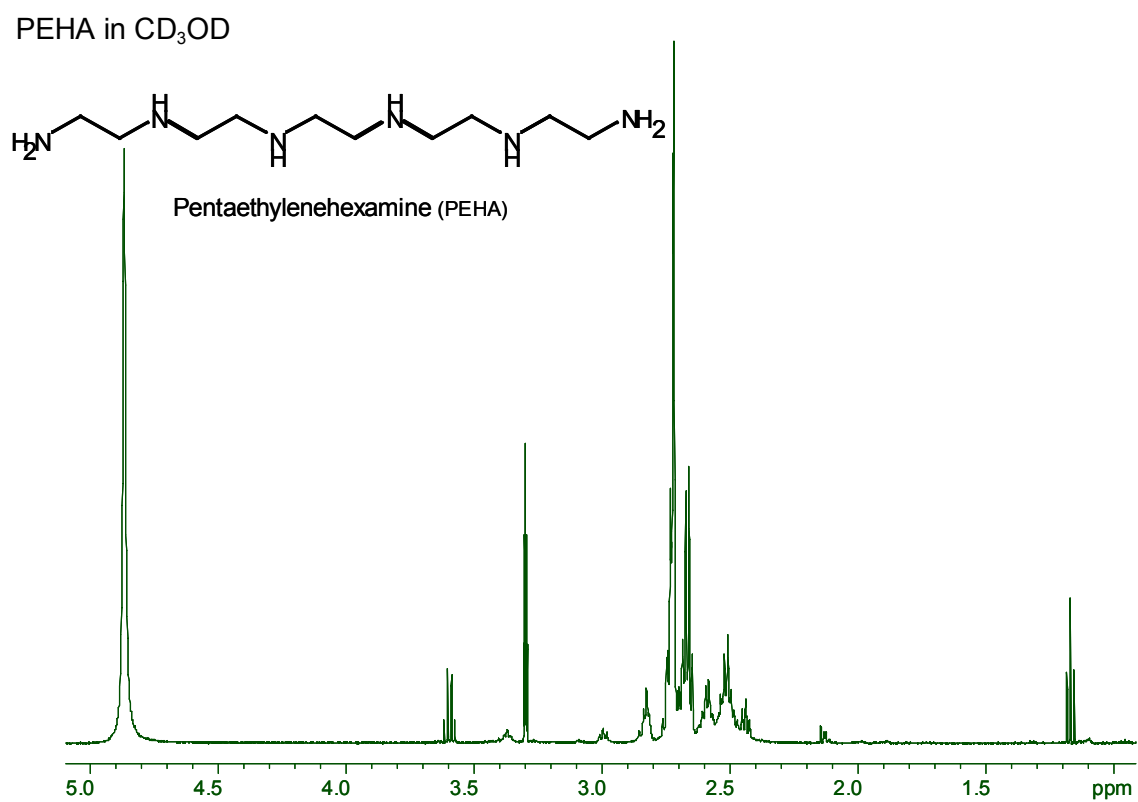


Figure 7. ¹H NMR of PEHA

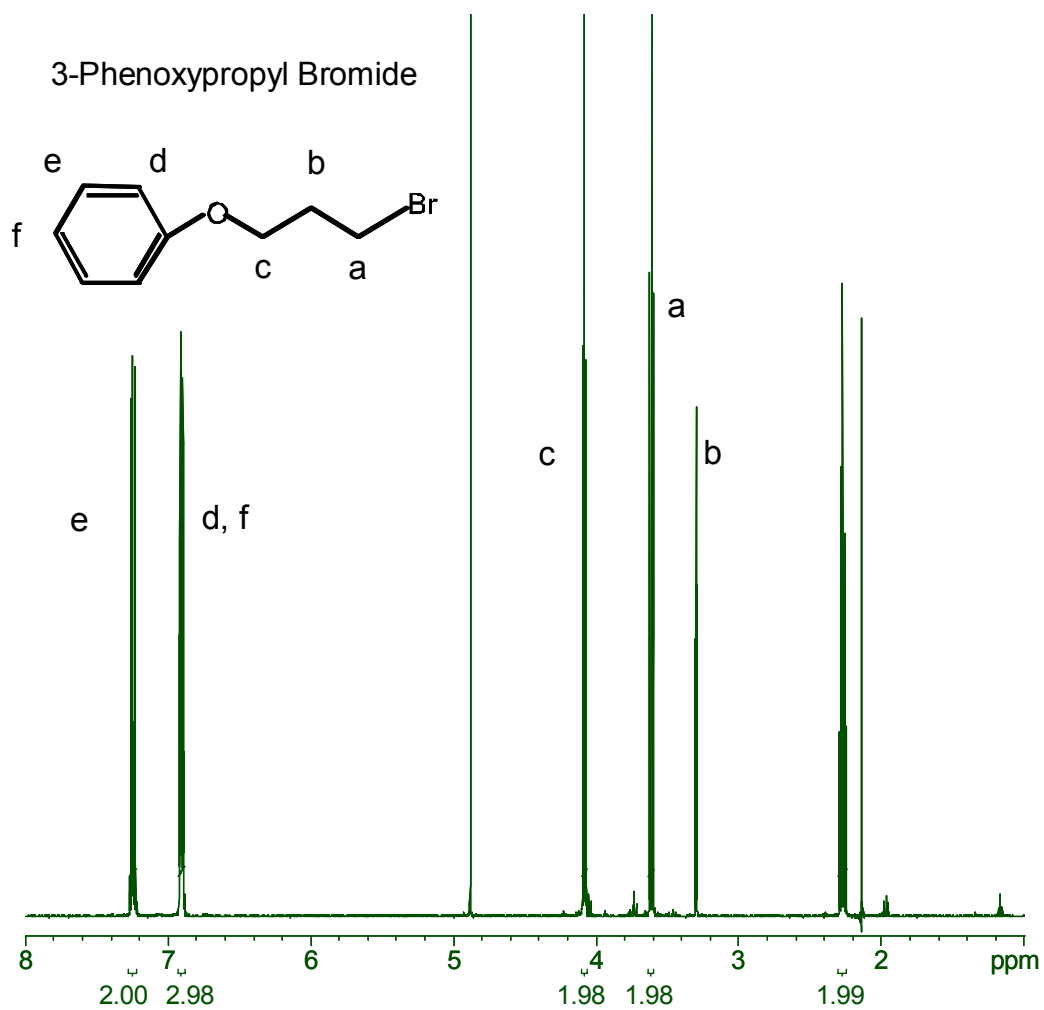


Figure 8. ¹H NMR of 3-PhOPrBr

3.4.1 Michael Addition with Acrylic Acid

Following the SN_2 alkylation reaction, the intermediate was reacted with acrylic acid. Several batches of UV-absorbing PEHA carrier ampholytes with 1 equivalent of acrylic acid were synthesized and then combined to produce a homogenous mixture. For the subsequent Michael additions, an aliquot of the combined batches were used.

The mixture was stirred and heated in a 1-L, 3-neck, round-bottom flask used for the alkylation reaction. While the mixture was coming to the temperature of 80 – 90 °C, 6% w/v (relative to PEHA) of deionized water from the Milli-Q system was added to the reaction mixture. Once a homogenous mixture was achieved, a mixture of 1 to 8 equivalents of acrylic acid and an additional 6% w/v (relative to PEHA) of deionized water was added dropwise to the reaction mixture at a rate of 1 drop every 8 seconds. Each drop of acrylic acid/water was thoroughly mixed before the next drop was added. The acrylic acid/water mixture was added over 6 hours. The reaction mixture was kept under N_2 atmosphere. The reaction flask was heated for an additional 12 – 18 hours between 90 – 95 °C. A possible reaction scheme is shown in Figure 9. The representative ^1H NMR spectra of the final product is shown in Figure 10 and that of the acrylic acid starting material is shown in Figure 11. The representative ^1H NMR spectrum of

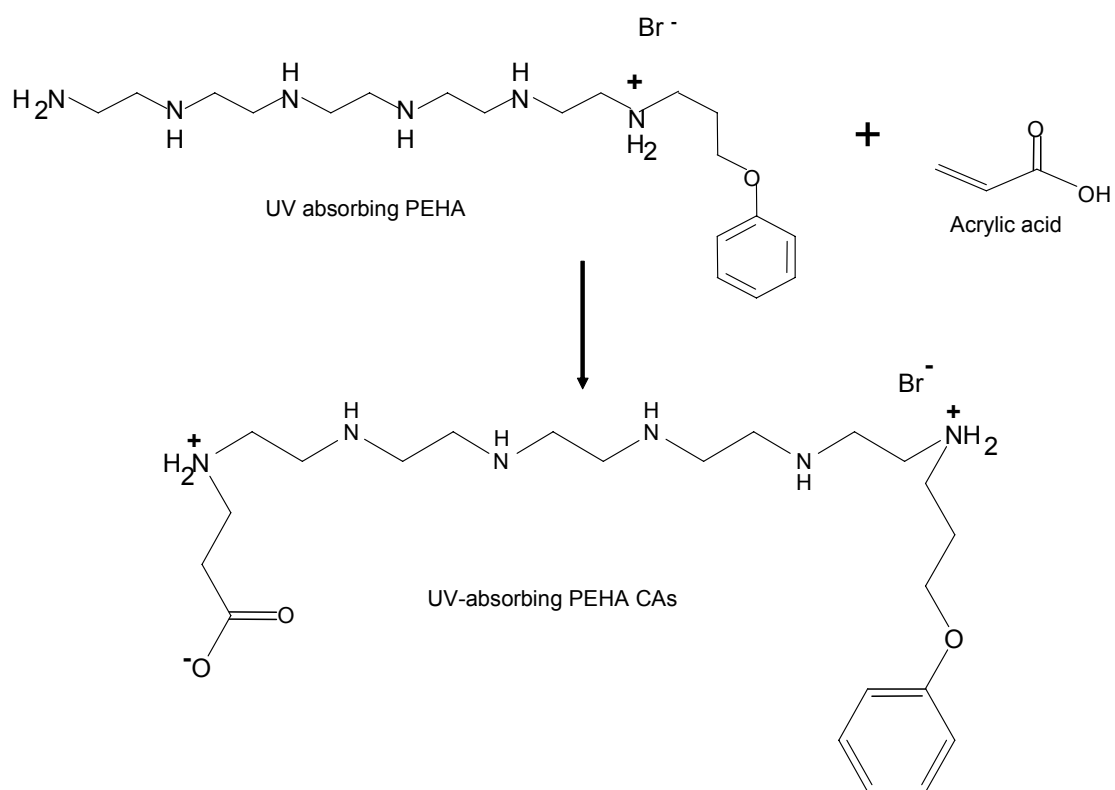


Figure 9. Reaction scheme of Michael's addition

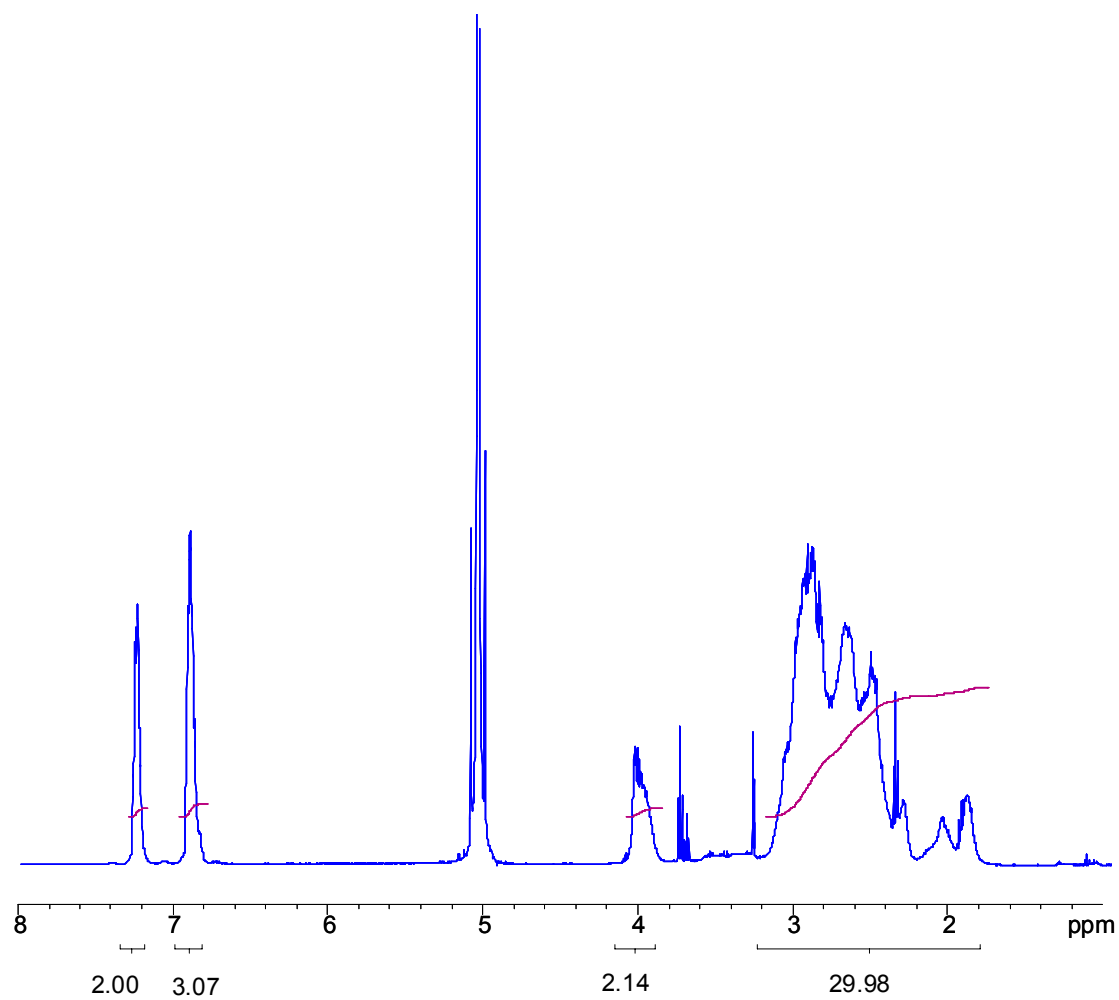


Figure 10. ^1H NMR of the final product of Michael's addition reaction using 1 equiv acrylic acid

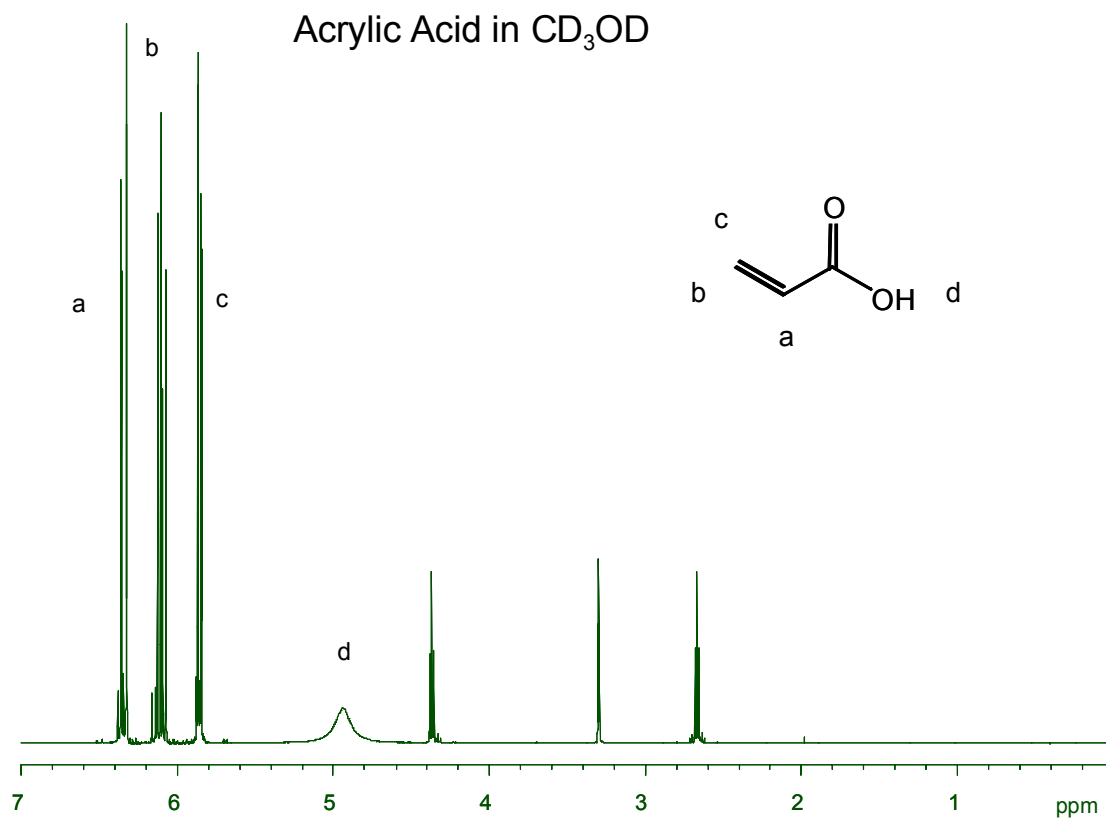


Figure 11. ¹H NMR of acrylic acid

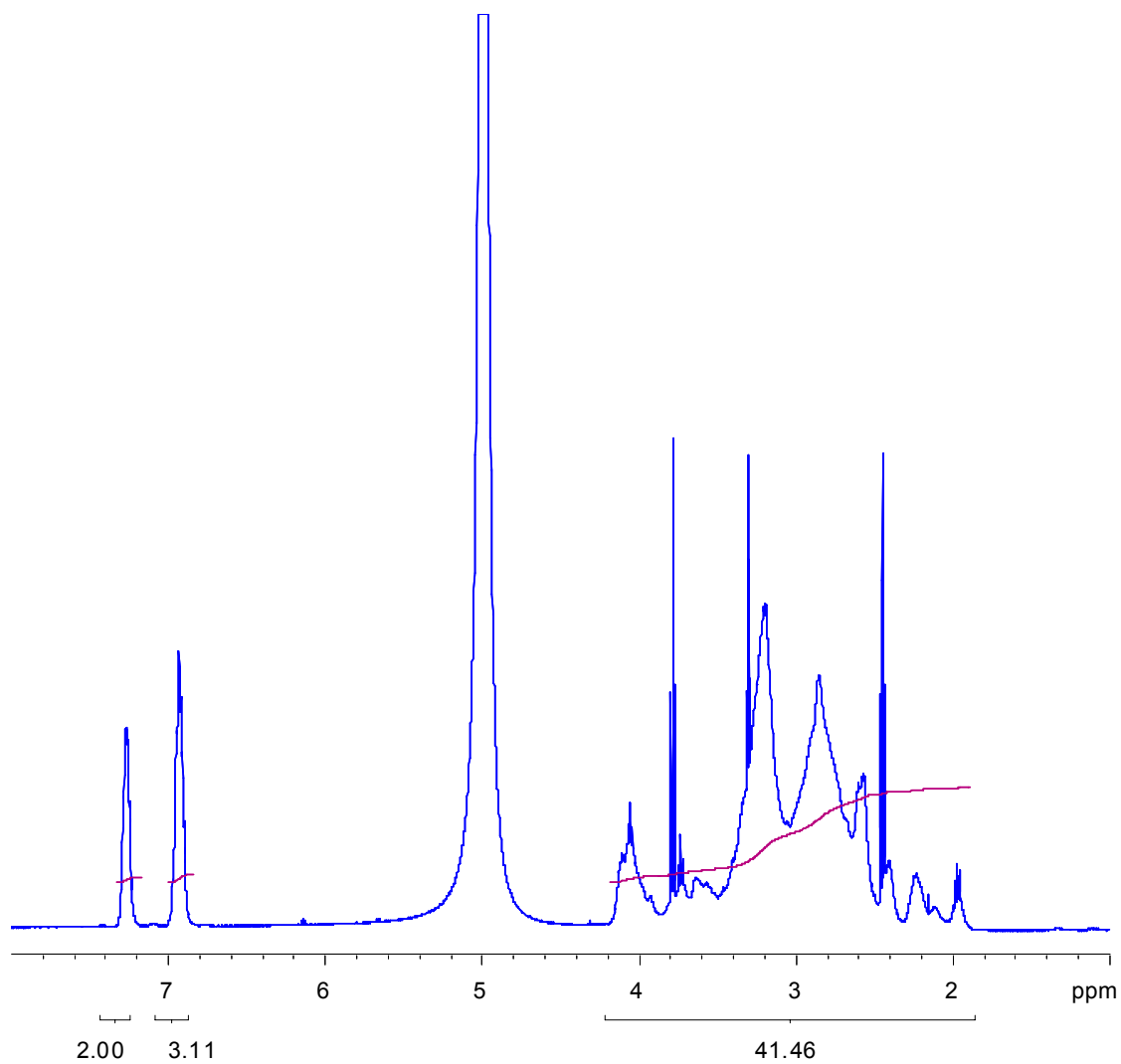


Figure 12. ^1H NMR of the final product of Michael's addition reaction using 3 equiv acrylic acid

the final product with unreacted acrylic acid is shown in Figure 12 (3 equiv of acrylic acid).

To determine the pI range of the UV-absorbing PEHA carrier ampholytes, a full-column imaging CIEF was performed. The analyte mixture was composed of 8 – 12% Pharmalytes pH 3-10; Arg and IDA as cathodic and anodic blockers; TYRA, DNS Asp, and/or DNS Asp, DNS Phe, MABA, TER, DOPA as pI markers; a minimum of 0.1% MC, and 0.75 μ M (final concentration in the well) of the UV-absorbing PEHA carrier ampholytes. The electropherograms for the UV-absorbing PEHA carrier ampholytes using 1 to 8 equivalents of acrylic acid are shown in Figures 13 to 20, respectively.

By increasing the number of equivalents of acrylic acid, the UV-absorbing carrier ampholytes were made more acidic as shown in Table 2. As more equivalents of acrylic acid were added, more of it was left unreacted, beginning with the addition of 3 equivalents of acrylic acid. The unreacted material produced ^1H NMR signals at 5.8, 6.1, and 6.3 ppm, which correspond to the protons on the α - and β - unsaturated carbons of acrylic acid.

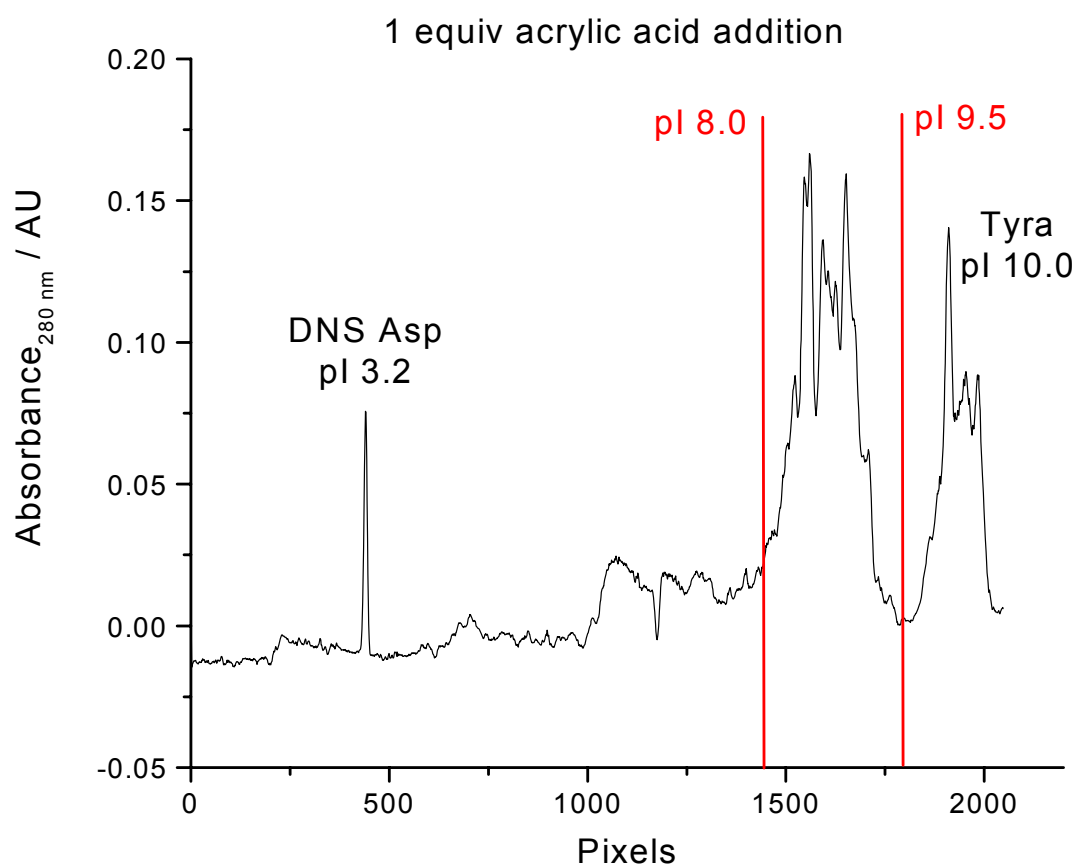


Figure 13. ICIEF trace of a homogenous mixture of UVCAAs using 1 equiv acrylic acid

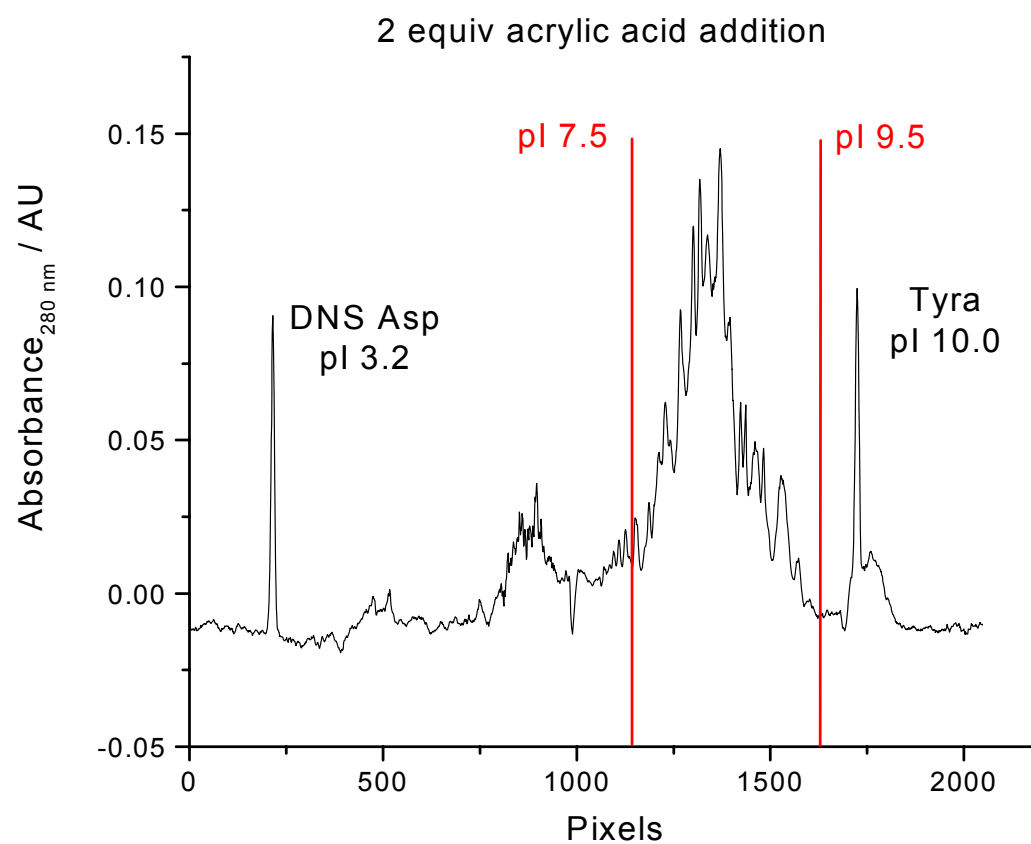


Figure 14. ICIEF trace of a homogenous mixture of UVCAAs using 2 equiv acrylic acid

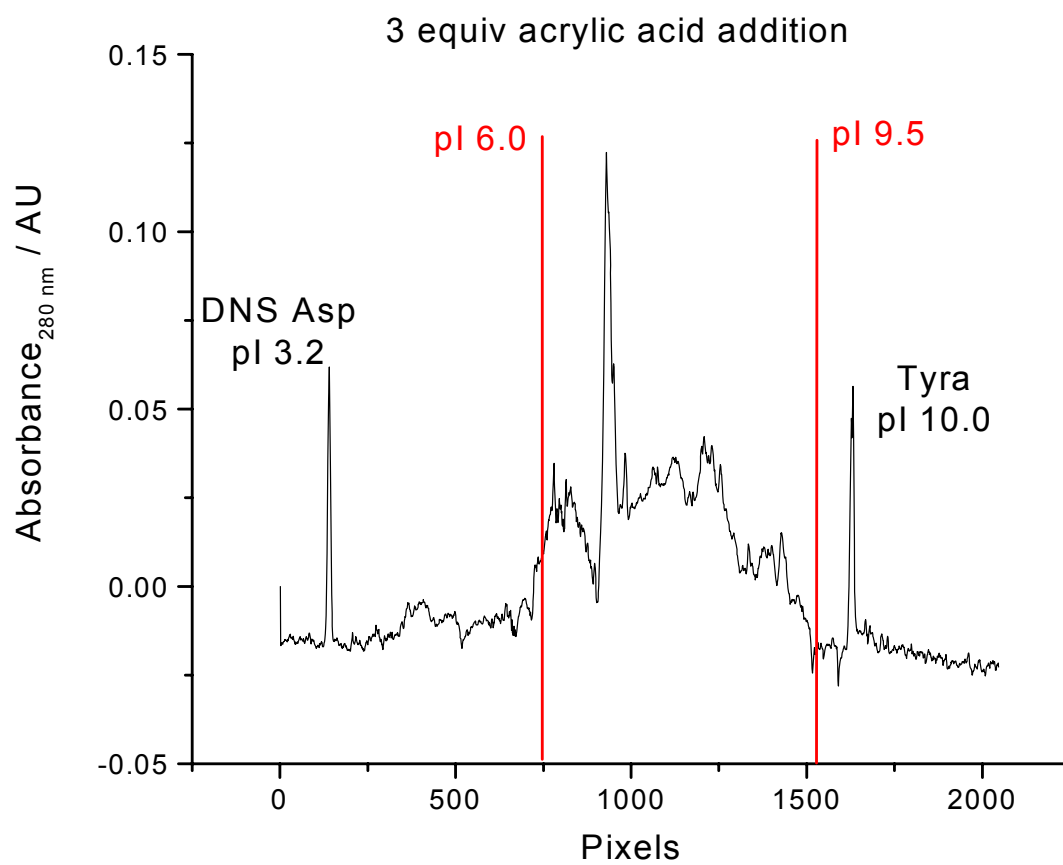


Figure 15. ICIEF trace of a homogenous mixture of UVCAs using 3 equiv acrylic acid

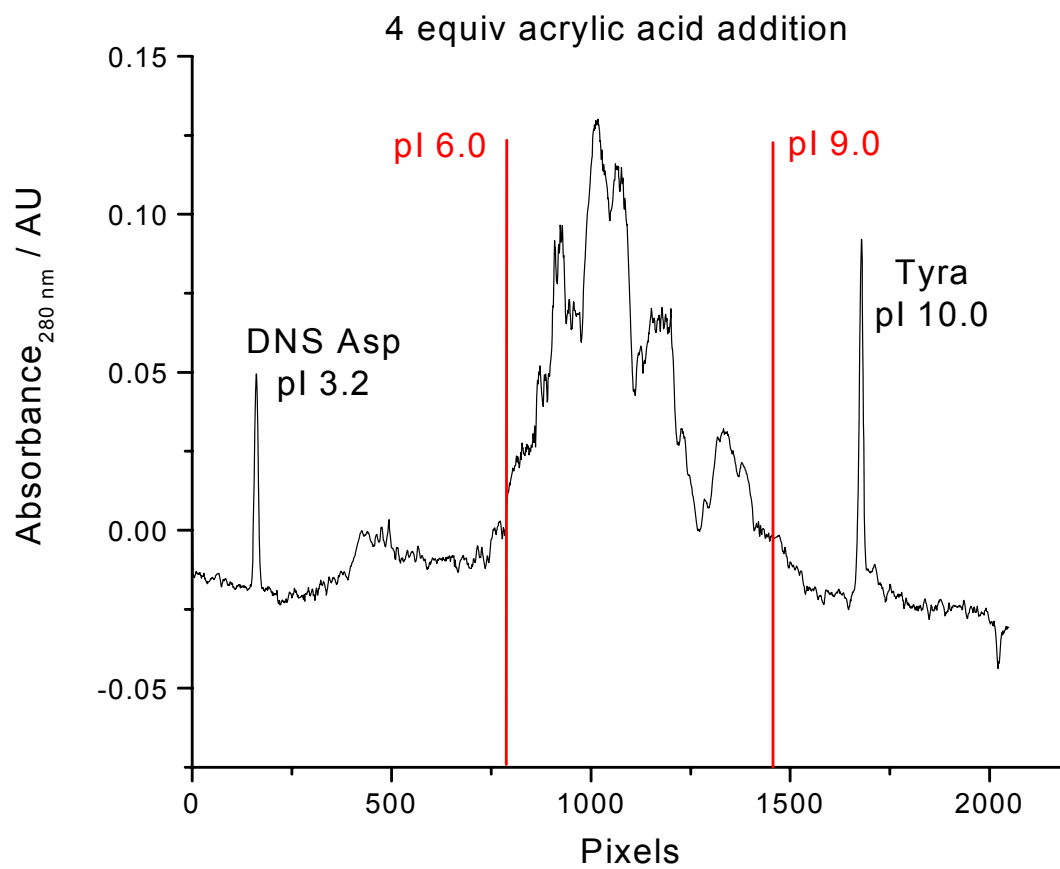


Figure 16. ICIEF trace of a homogenous mixture of UVCAs using 4 equiv acrylic acid

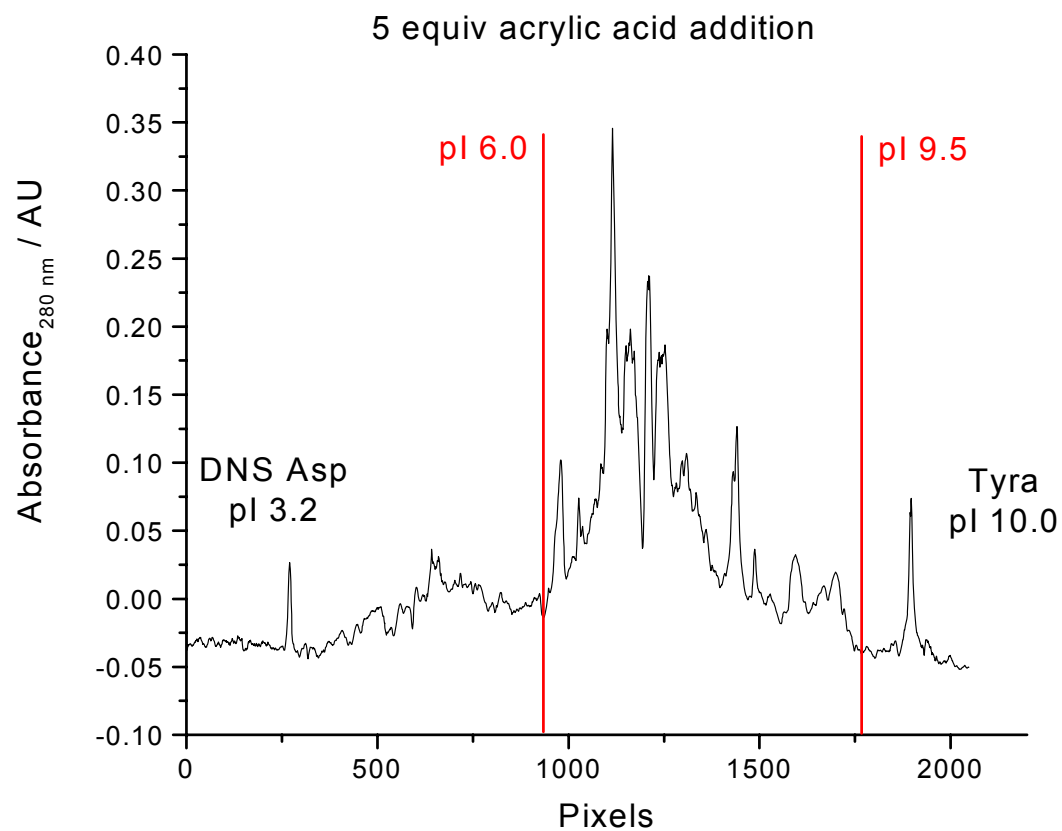


Figure 17. ICIEF trace of a homogenous mixture of UVCAAs using 5 equiv acrylic acid

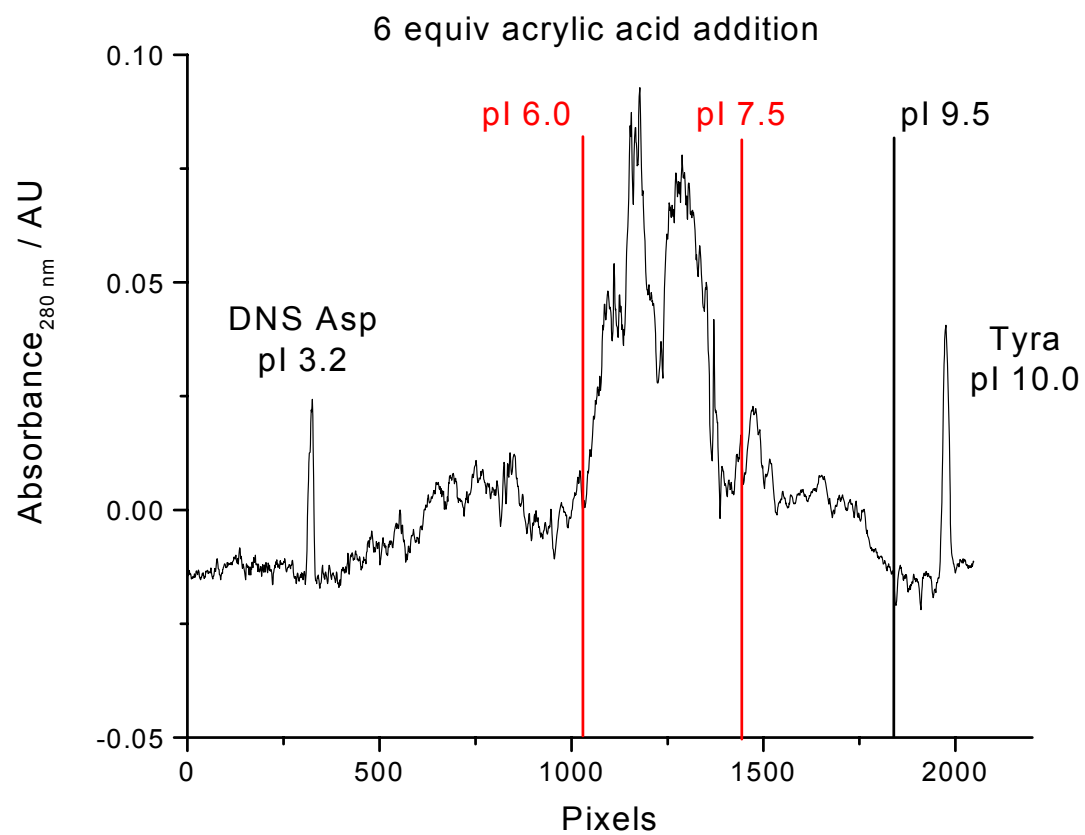


Figure 18. ICIEF trace of a homogenous mixture of UVCAAs using 6 equiv acrylic acid

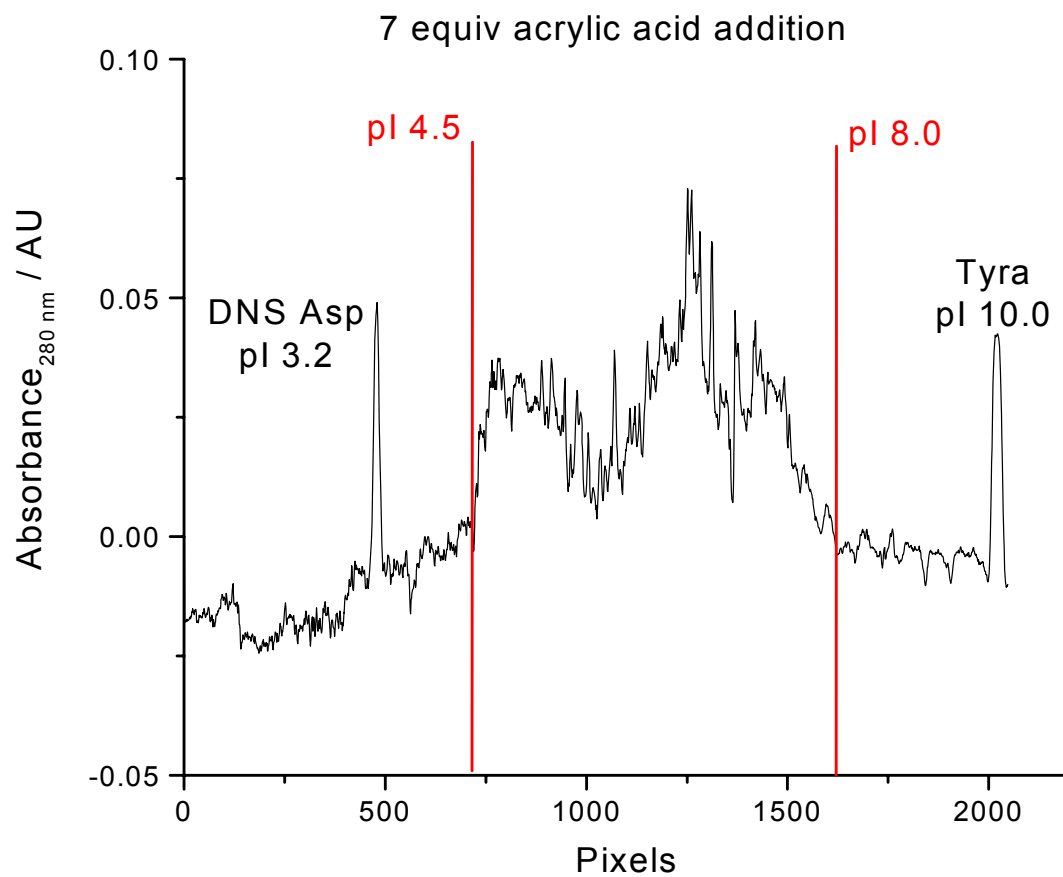


Figure 19. ICIEF trace of a homogenous mixture of UVCAs using 7 equiv acrylic acid

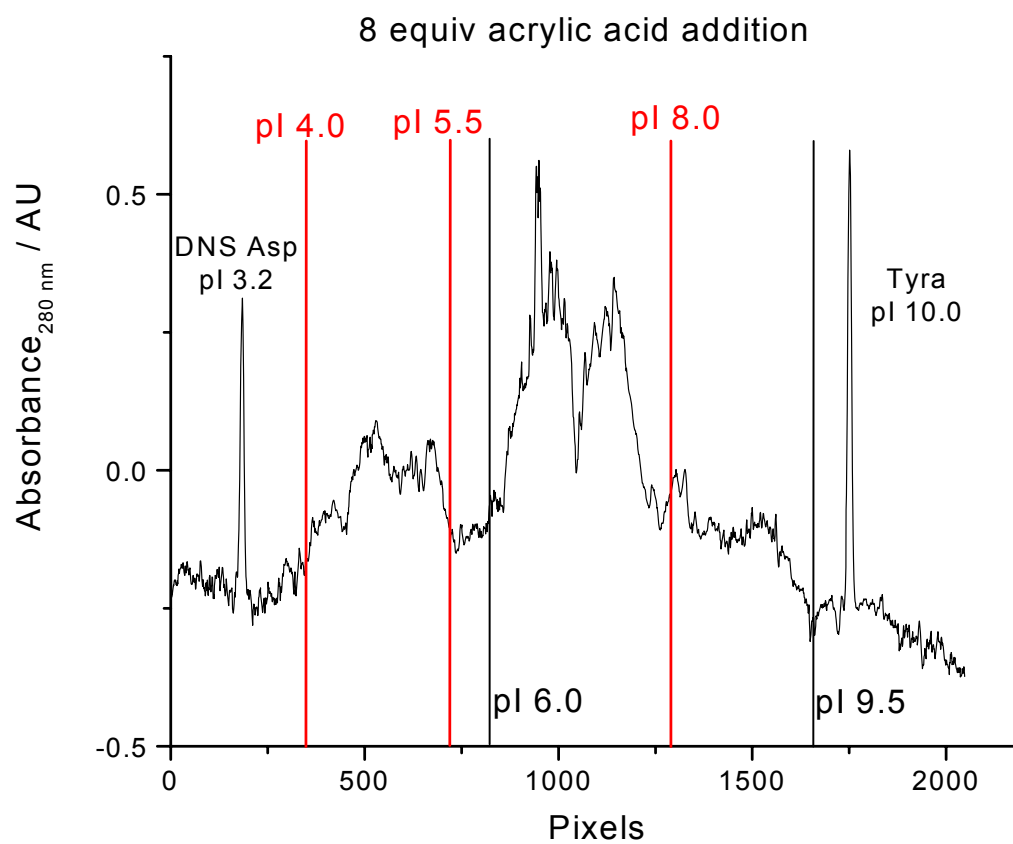


Figure 20. ICIEF trace of a homogenous mixture of UVCAAs using 8 equiv acrylic acid

Table 2. pI range of major and minor components for reactions with acrylic acid

# of equiv of acrylic acid	pI range* of major components	pI range* of minor components
1	8.0 – 9.5	N/A
2	7.5 – 9.2	N/A
3	6.0 – 9.5	N/A
4	6.0 – 8.7	N/A
5	6.0 – 8.0	N/A
6	6.0 – 7.5	N/A
7	4.5 – 7.7	N/A
8	6.0 – 7.7	3.5 – 6.0

* pI's were calibrated with two pI markers, DNS Asp (pI = 3.2) and Tyra (pI = 10.0)

Table 3. pI range of major and minor components for reactions with acrylic acid + itaconic acid

# of equiv of acrylic acid	# of equiv of itaconic acid	pI range* of major components	pI range* of minor components
1	1	5.8 – 6.6 6.8 – 9.8	N/A
1	2	5.7 – 9.8	N/A
7	1	6.0 – 6.8 6.9 – 9.3	N/A
6	2	6.0 – 8.3	3.3 – 6.0 8.3 – 9.8

* pI's were calibrated with two pI markers, DNS Asp (pI = 3.2) and Tyra (pI = 10.0)

3.4.2 Michael Addition with Itaconic Acid

1 to 2 equivalents of itaconic acid were added to the combined batch containing 1 equivalent of acrylic acid. This Michael addition was performed in the same manner as previously. The reaction mixture was heated to 92 – 94 °C in a N₂ environment for 4 days. The reaction scheme depicting a possible product is shown in Figure 21. The ¹H NMR spectra of the final product containing 1 equivalent of itaconic acid and acrylic acid each is shown in Figure 22. The ICIEF electropherograms of the two intermediate carrier ampholytes are shown in Figures 23 and 24.

Michael additions to the rest of the amino groups on the PEHA backbone were performed by adding 5 to 6 more equivalents of acrylic acid. The reaction mixture was heated to 80 °C in a N₂ environment for 12 hours. The ¹H NMR of the final product containing 2 equivalents of itaconic acid and 6 equivalents of acrylic acid is shown in Figure 25. The electropherograms for the UV-absorbing PEHA carrier ampholytes using 1 or 2 equivalents of itaconic acid with 7 or 6 equivalents of acrylic acid, respectively, are shown in Figures 26 and 27. Table 3 summarizes the pI ranges of the major and minor compounds of the different carrier ampholyte batches.

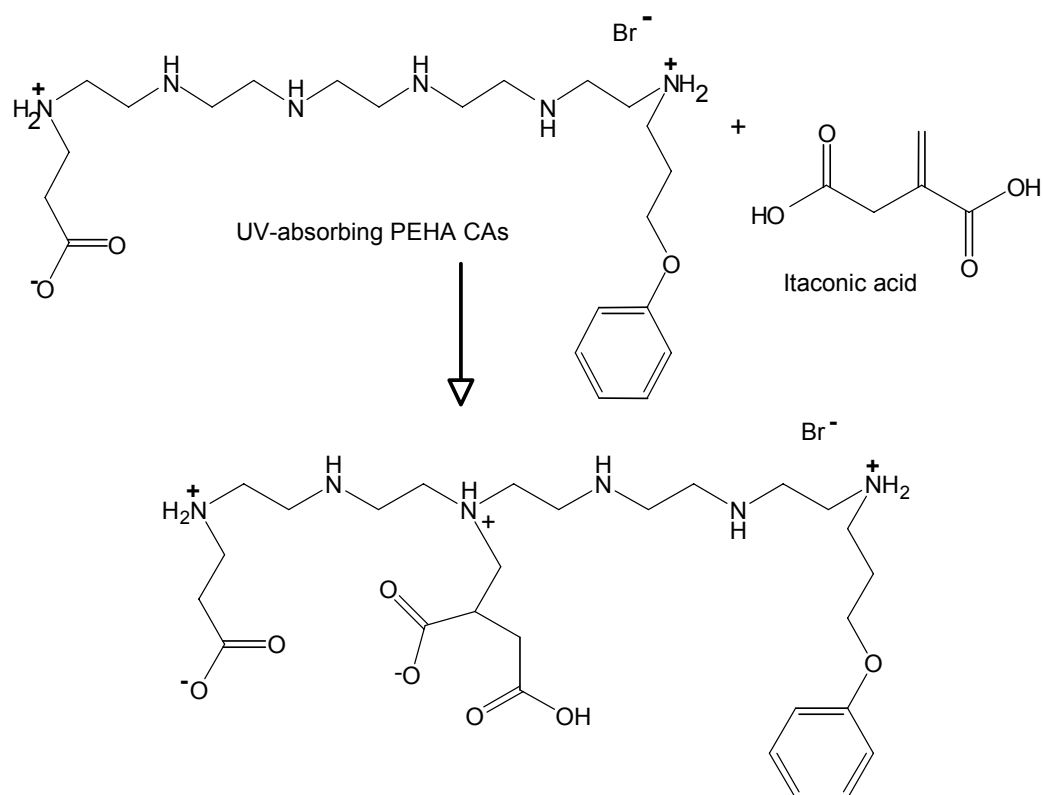


Figure 21. Reaction scheme of Michael's addition using 1 equiv itaconic acid + 1 equiv acrylic acid

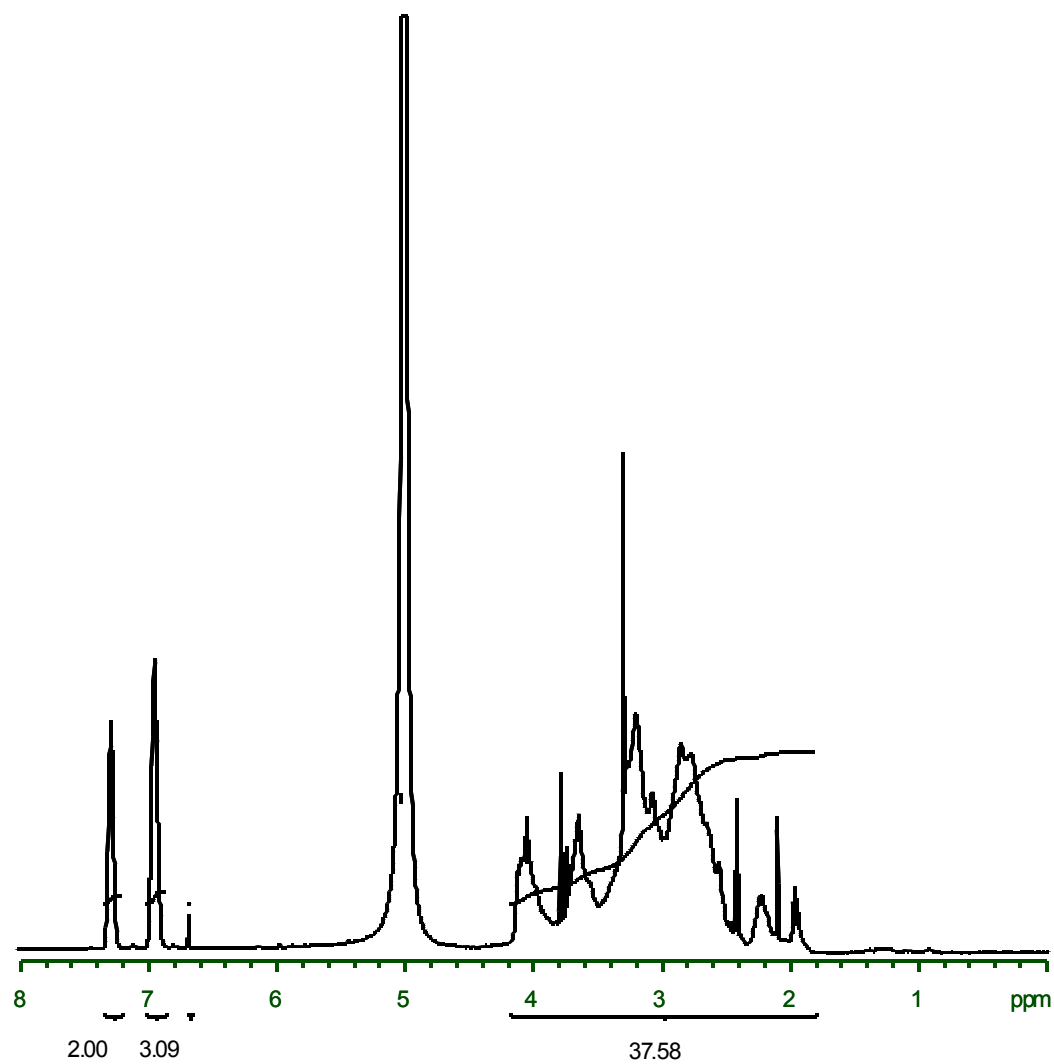


Figure 22. ^1H NMR of intermediate product of Michael's addition reaction using 1 equiv itaconic acid + 1 equiv acrylic acid

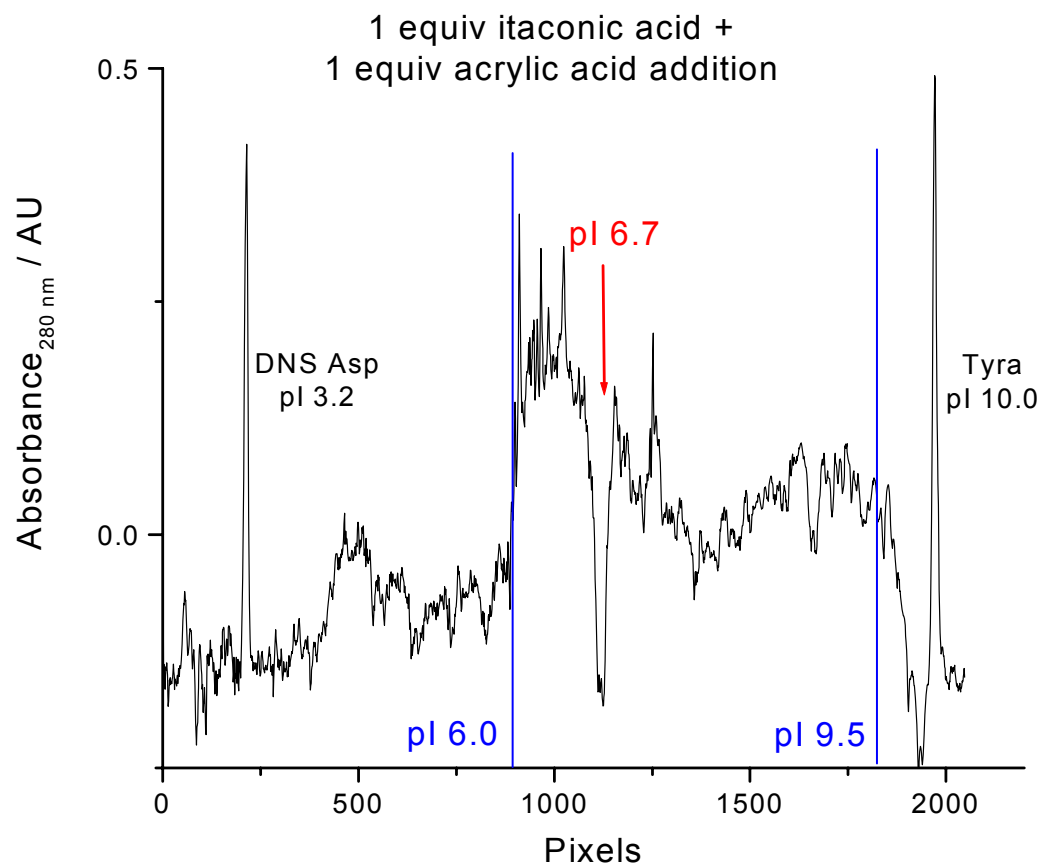


Figure 23. ICIEF trace of intermediate UVCAs containing 1 equiv itaconic acid + 1 equiv acrylic acid

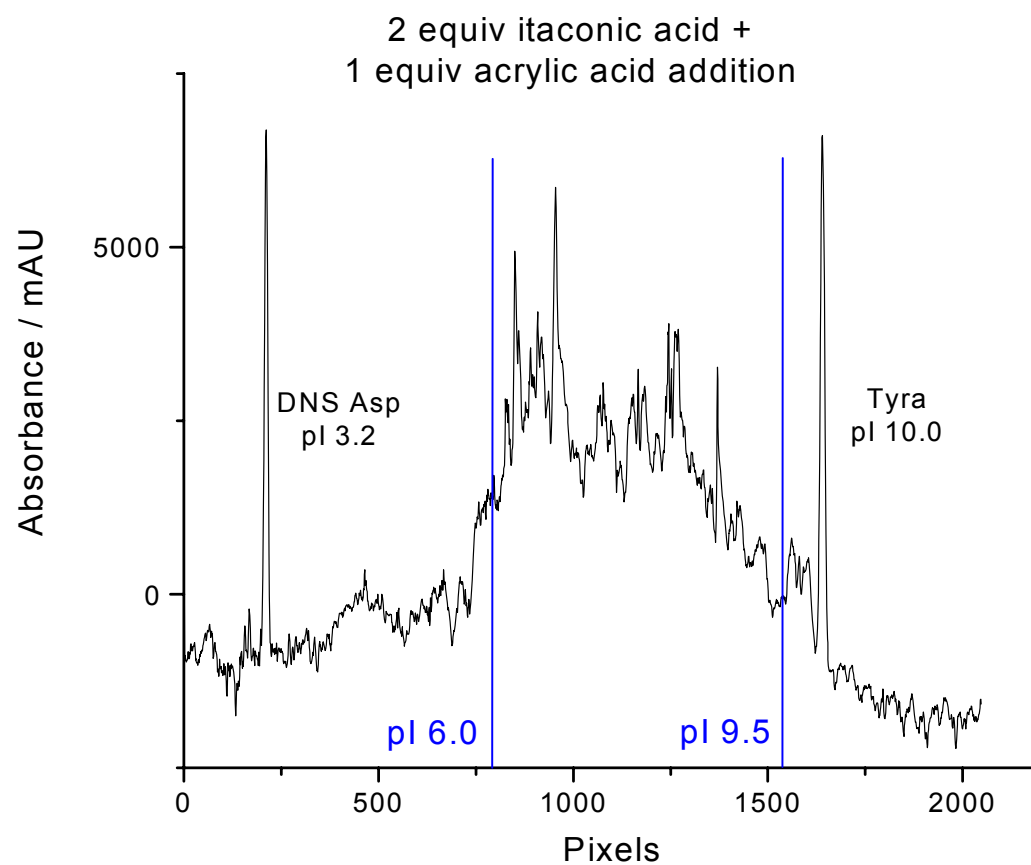


Figure 24. ICIEF trace of intermediate UVCAs containing 2 equiv itaconic acid + 1 equiv acrylic acid

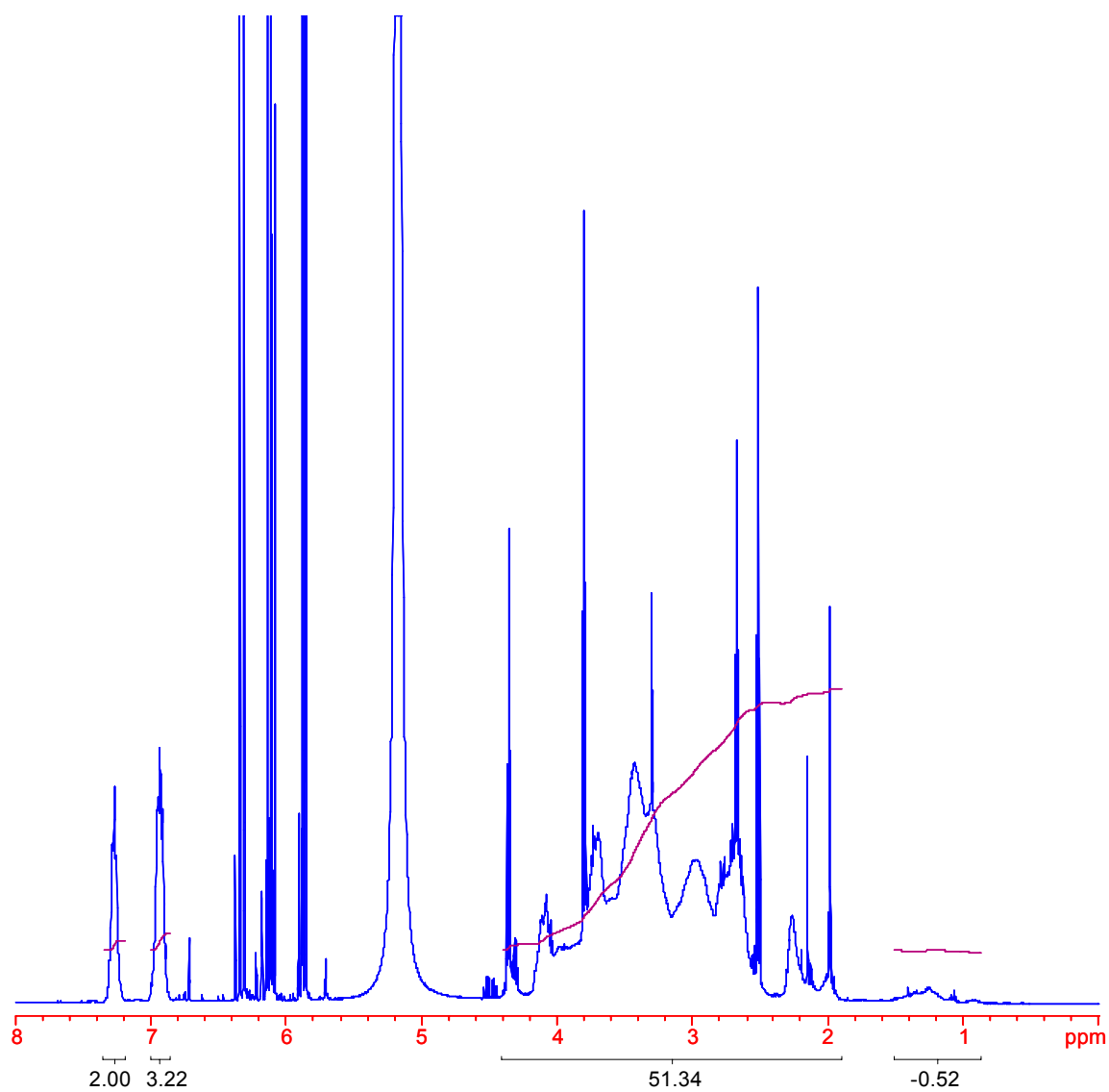


Figure 25. ^1H NMR of final product containing 2 equiv itaconic acid + 6 equiv acrylic acid

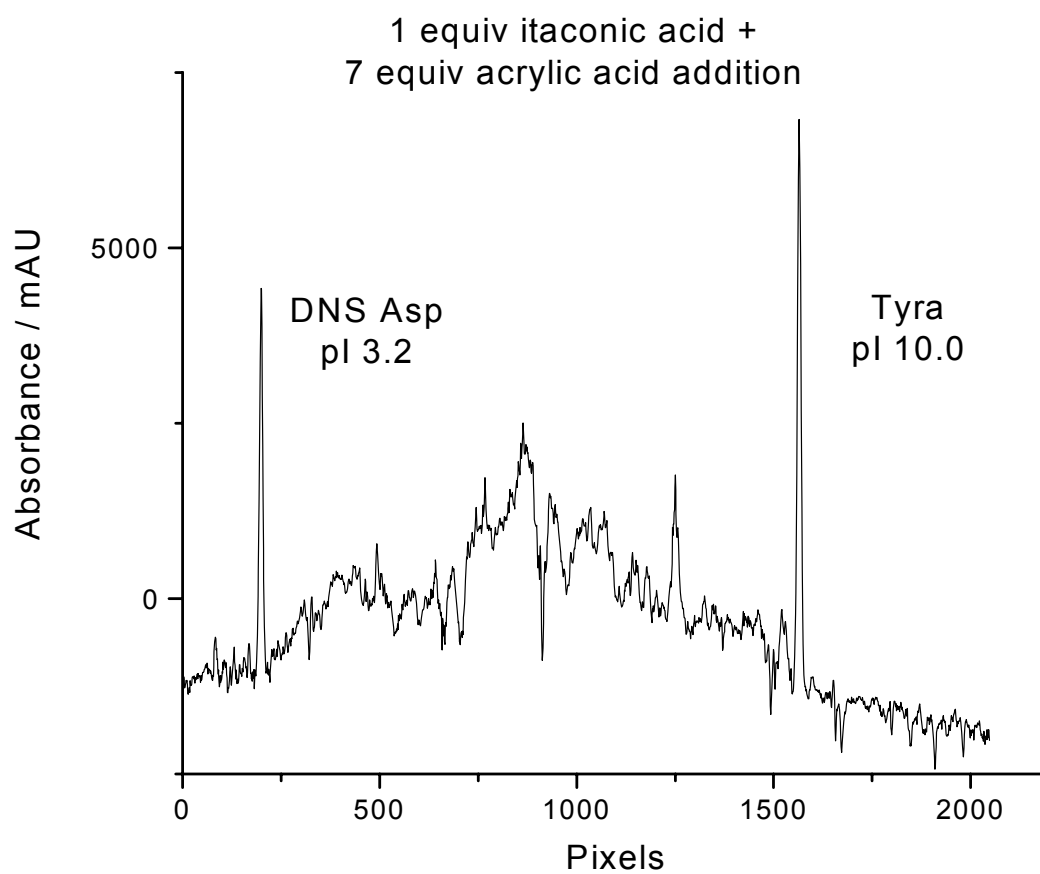


Figure 26. ICIEF trace of final product containing 1 equiv itaconic acid + 7 equiv acrylic acid

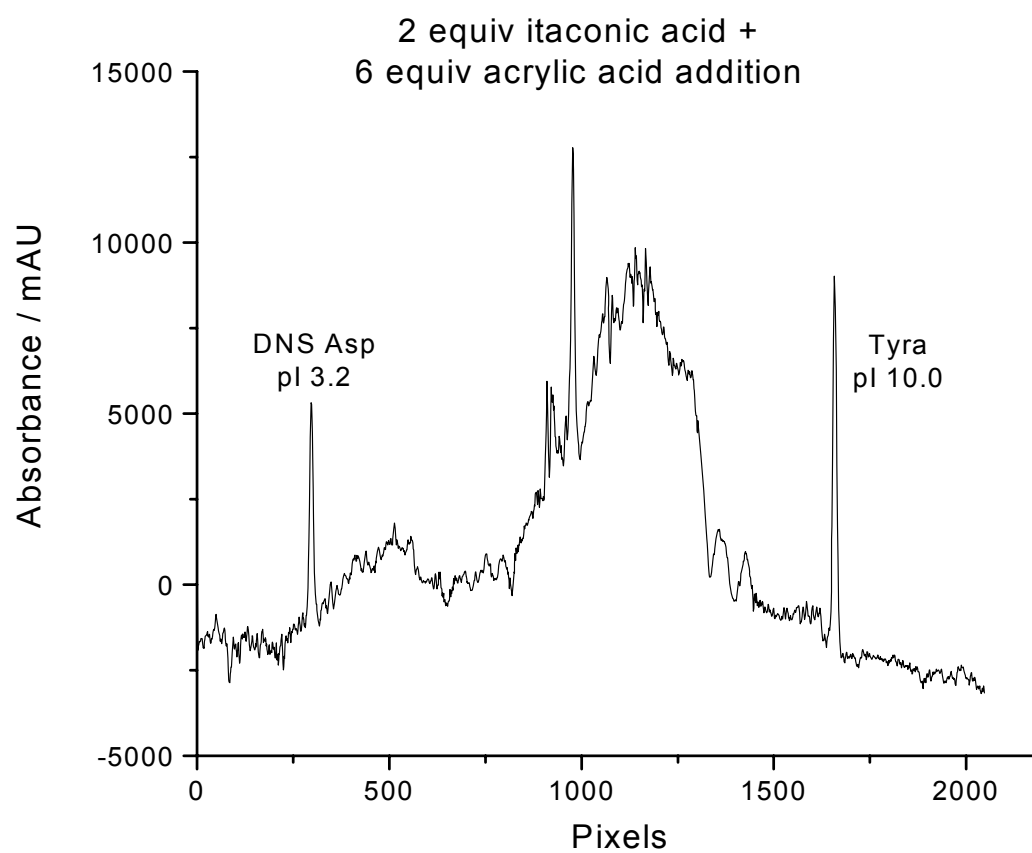


Figure 27. ICIEF trace of final product containing 2 equiv itaconic acid + 6 equiv acrylic acid

Carrier ampholytes obtained by reacting poly(ethylene imines) and acrylic acid have exhibited a conductivity minimum in the pH range 5.5 – 6.5 due to the lack of available carrier ampholytes in that region [24]. The reported pKa values of itaconic acid are $pK_{a1}=3.85$ and $pK_{a2}=5.45$. Addition of only 1 equivalent of itaconic acid to the UV-absorbing PEHA carrier ampholytes greatly improved the coverage of the pI range to 5.8 – 9.8, compared to the 2 equivalents of acrylic acid carrier ampholytes which had a pI range of 7.5 – 9.2. However, there is a gap between pI 6.6 – 6.8. Therefore, an additional equivalent of itaconic acid was added, which filled in the pI gap from the previous batch. Subsequently, 6 equivalents of acrylic acid were added to couple with the rest of the unreacted amino groups. This reaction produced UV-absorbing carrier ampholytes in the pI range 3.3 – 9.8, with the major carrier ampholytes in the 6.0 to 8.3 range.

3.5 Reproducibility of the Alkylation and Michael Reactions

Good reproducibility of the intermediates and final products was confirmed by comparing the ^1H NMR spectra of two 5-g batches, two 50-g batches, and one 100-g batch that were synthesized. A representative comparison of the ^1H NMR spectra of the small-scale and large-scale batches of the alkylation reaction and the Michael additions are shown in Figures 28 and 29, respectively.

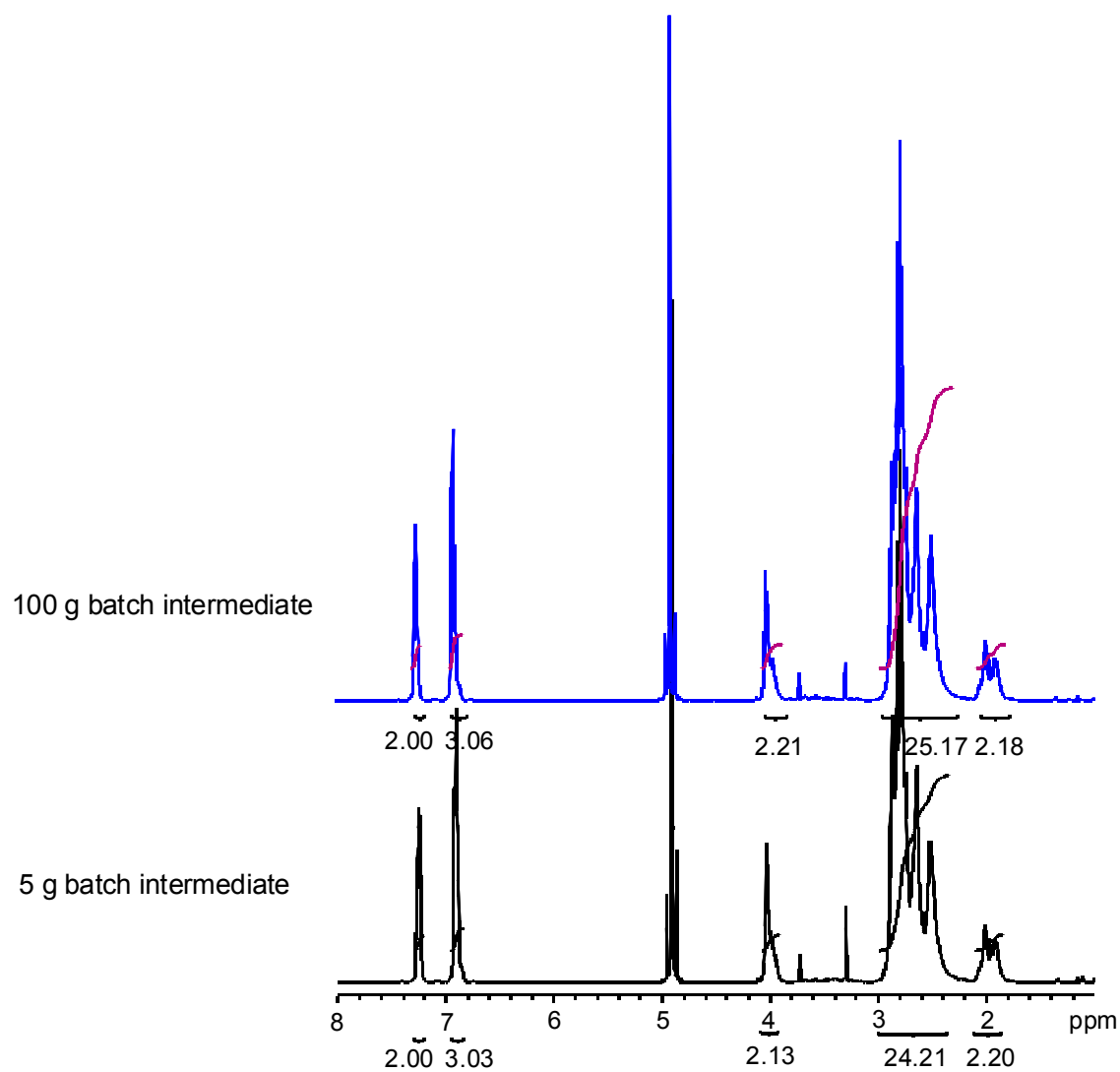


Figure 28. Comparison of 5-g and 100-g batches of intermediates from the alkylation reaction using 3-PhOPrBr

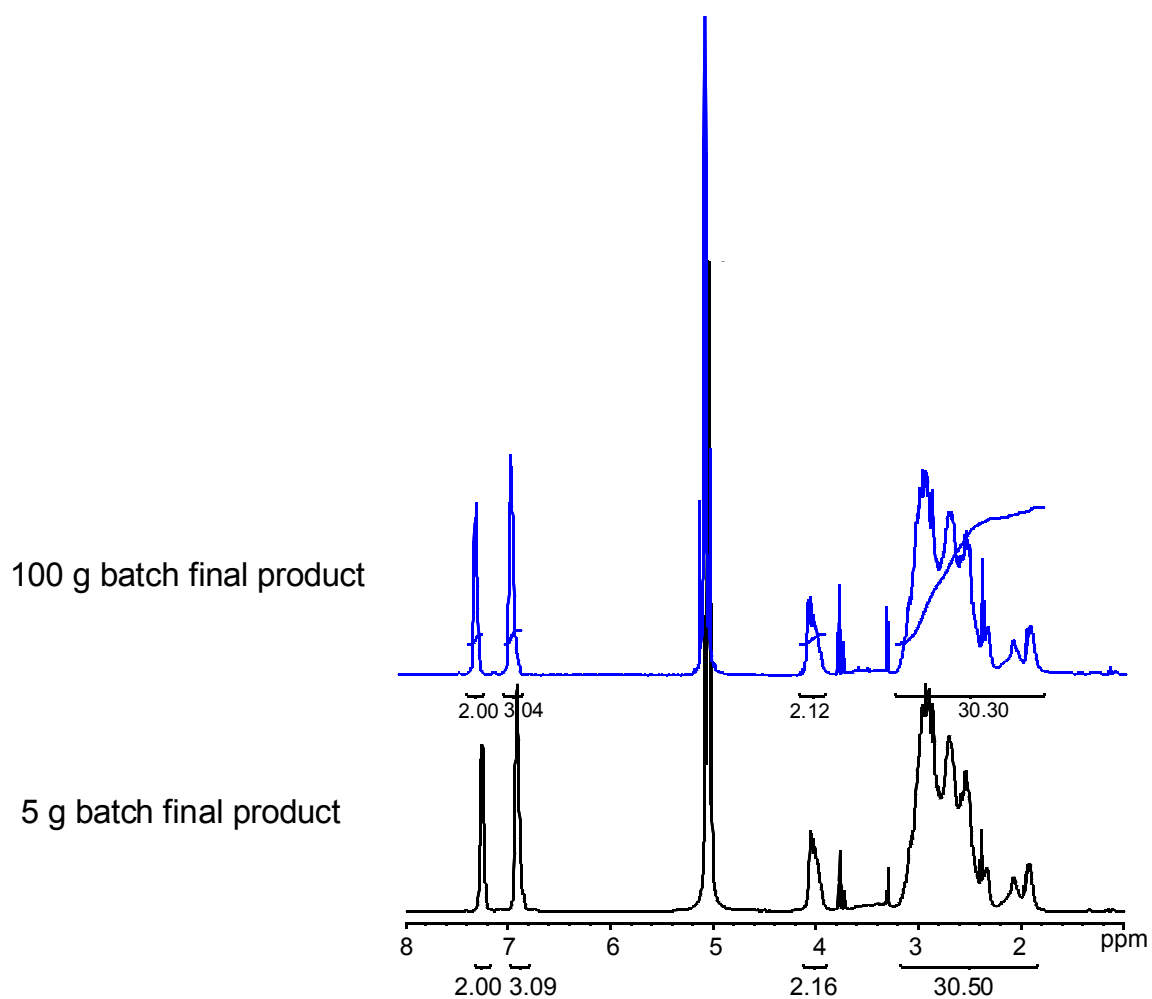


Figure 29. Comparison of 5-g and 100-g batches of PEHA UVCAs containing 1 equiv acrylic acid

3.6 Binary IET Separations

The UVCAs were desalted and purified by running binary IET separations in the modified BF200IET Gradiflow unit.

3.6.1 Desalting and Purification

First, the cartridge for the modified BF200IET unit was prepared using an IDA/PVA anodic restriction membrane (pH \sim 2), a Glu/PVA separation membrane (pH 2.6 – 3.4), and an Imino/PVA cathodic restriction membrane (pH $>$ 10). The anode compartment was filled with 30 mM MSA and the cathode compartment was filled with 150 mM NaOH. 5.658 g of UVCAs with 2 equivalents of itaconic acid and 6 equivalents of acrylic acid were dissolved in 3.5 L of deionized water to produce a clear, yellow solution. The sample was fed into the downstream compartment (located next to the anode compartment), while 50 mL of 0.1 mM Na₂SO₄ were loaded into the upstream compartment (located next to the cathode compartment). Na₂SO₄ was used to ensure initial conductivity throughout the four chambers. All compartments were cooled with ice water. To stay within a 75 W maximum power limit, the power supply was initially set to 500 mA and 150 V.

The IET separation was run in a pass-by-pass mode with each pass lasting less than 2 hours. Enough material had transferred to the upstream (collection)

compartment in 2 passes because the upstream sample turned a very bright, clear, yellow color. The salt moved out first; SO_4^{2-} moved towards the anode and Na^+ moved towards the cathode. Additionally, the catalyst used in the original alkylation reaction was moved out into the electrolyte compartments. Throughout the separation, the current ranged from 217 – 358 mA while the potential stayed at 150 V. Although the power was only between 33 – 54 W, much of the material had moved to the collection stream. As the UVCAAs moved into the upstream compartment, the conductivity went from 27 μS to 62 μS , while the conductivity in the downstream decreased from 207 μS to 122 μS . This change in conductivity was due to the increase in the amount of ampholytes in the upstream and the decrease in the amount of ampholytes in the downstream. In addition, as the separation progressed, bromine was formed evidenced by the smell and the yellow color of the anolyte. Therefore, the anolyte and catholyte were constantly replaced with fresh solutions. After the IET was completed, the content of the upstream was rotovapped under high vacuum yielding 0.442 g of material. The material was rediluted into 6 mL of water for the preparation of the iCIEF sample.

The iCIEF sample contained: 4% Pharmalytes, pH 3-10; 21 mM ARG; 12.5 mM IDA; 83 μM TYRA; 15.6 μM DNS Asp; 22.5 μM DNS Phe; 31.3 μM MABA; 50 μM TER; 83 μM DOPA; 0.2 % MC; and 40.0 μL of the prepared analyte from the IET run. The desalting time was 0.5 min at 500 V and the focusing time was 10

min. Figure 30 shows the comparison of the electropherograms from the iCE280 for the upstream (collection) sample and the original sample.

By using the IDA/PVA and Glu/PVA buffering membranes, unreacted acrylic acid ($pK_a \sim 3$) and other weak acids will stay between these membranes ($2 < pH < 3.4$). Since the upstream is collected and used for the next run, a desalted and semi-purified sample is obtained for the subsequent IET run.

Following the first purification IET separation, the next one was set up in the same manner using the Glu/PVA anodic restriction membrane, the Imino/PVA separation membrane, and the Q/PVA cathodic restriction membrane ($pH \sim 12$). The sample collected from the first purification step was diluted to 400 mL with deionized water. This was loaded into the upstream (feed) while 20 mL of 0.1 mM Na_2SO_4 were loaded into the downstream (collection). The IET was run in a pass-by-pass mode again with the same settings (500 mA, 140 V) and the current, potential, and power were monitored throughout the separation as shown in Figure 31.

As the potential was kept constant, the current initially dropped until the first pass was completed, indicating that the sample was desalted. The current

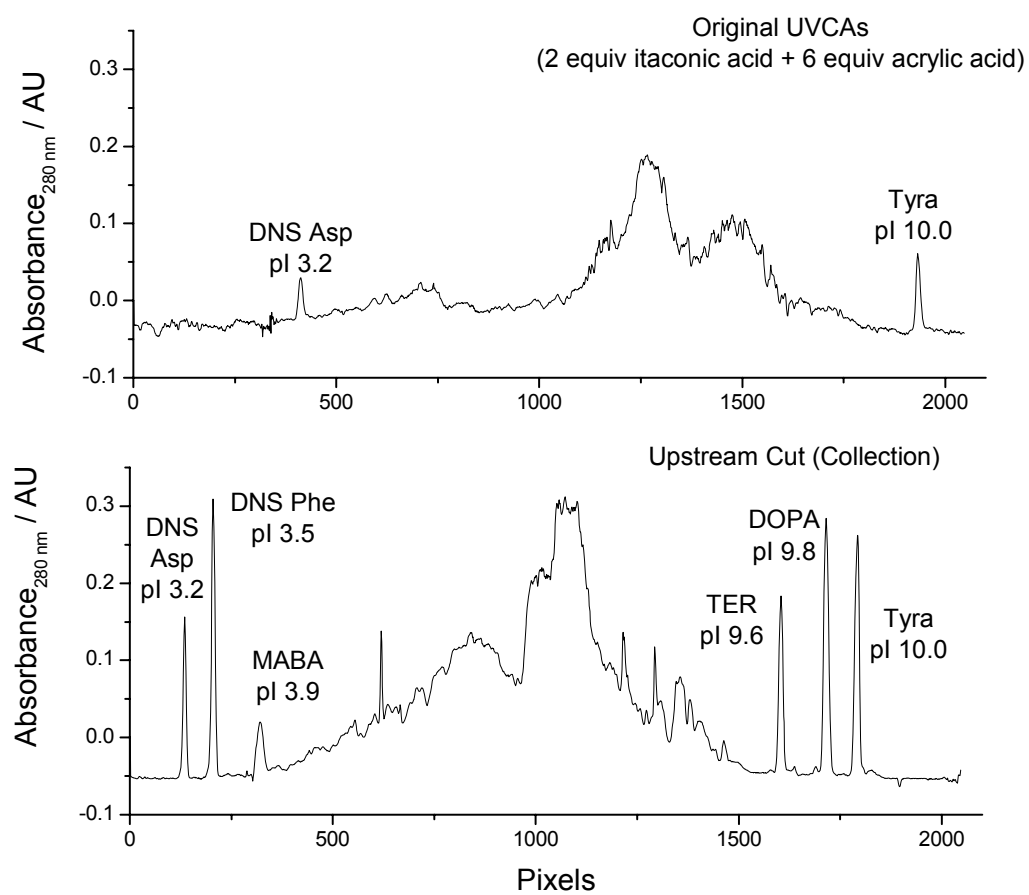


Figure 30. Comparison of the electropherograms for the upstream (collection) sample and the original UVCA

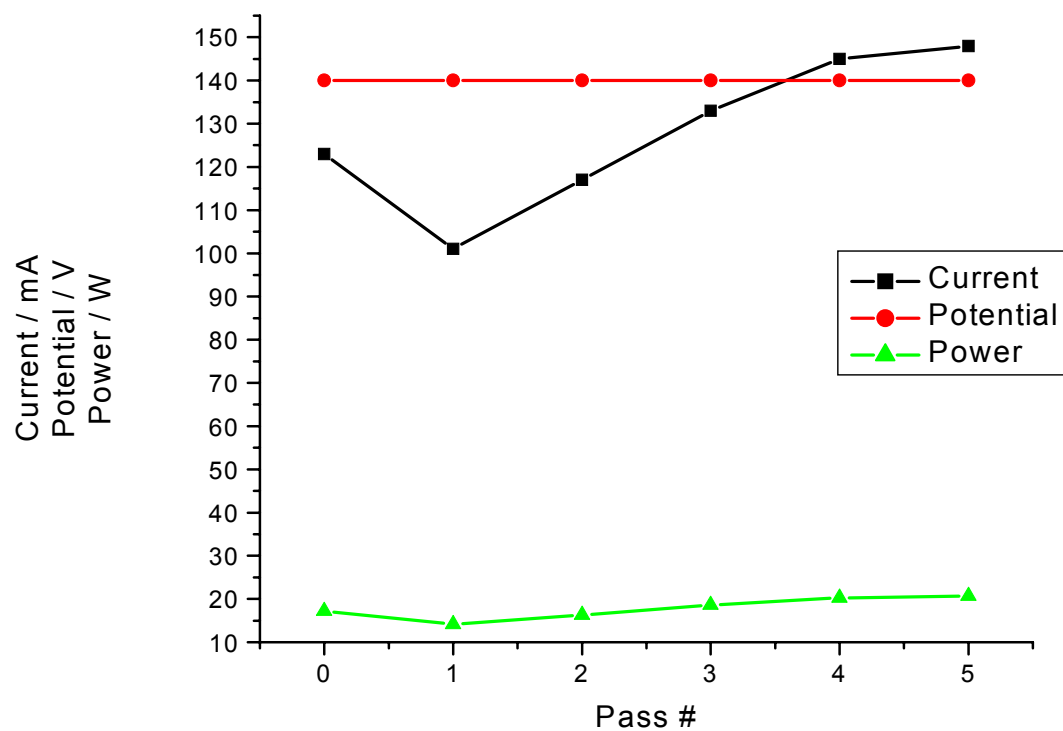


Figure 31. Plot of the current, potential, and power of the IET separation in pass-by-pass mode

increased after the first pass and continued to increase until the fourth pass, when it became steady. The transfer of UVCAAs from the upstream to the downstream was indicated by the increase in the current between the first and fourth passes. Once all the ampholytic material was transferred, the current leveled off. The power was kept relatively low, near 20 W.

Each pass was just under 26 minutes and 5 passes completed the IET separation. Figure 32 shows the ICIEF traces of the UV-transparent carrier ampholytes (blank sample) and the upstream cut (feed). This shows that all UVCAAs (0.4 g) moved to the downstream (collection) chamber in less than 2.5 hours. The ICIEF trace of the downstream sample, shown in Figure 33, shows that the UVCAAs were collected. Similarly to the previous IET separation, weak bases stayed in the upstream because Imino/PVA and Q/PVA membranes bracketed that chamber.

3.7 Summary

UV-absorbing PEHA-based carrier ampholytes were synthesized via an anti-Markovnikov Michael addition by first converting PEHA into a UV-absorbing amine and subsequently attaching 1 – 8 equivalents of acrylic acid and/or 1 – 2 equivalents of itaconic acid. Each intermediate and final product was

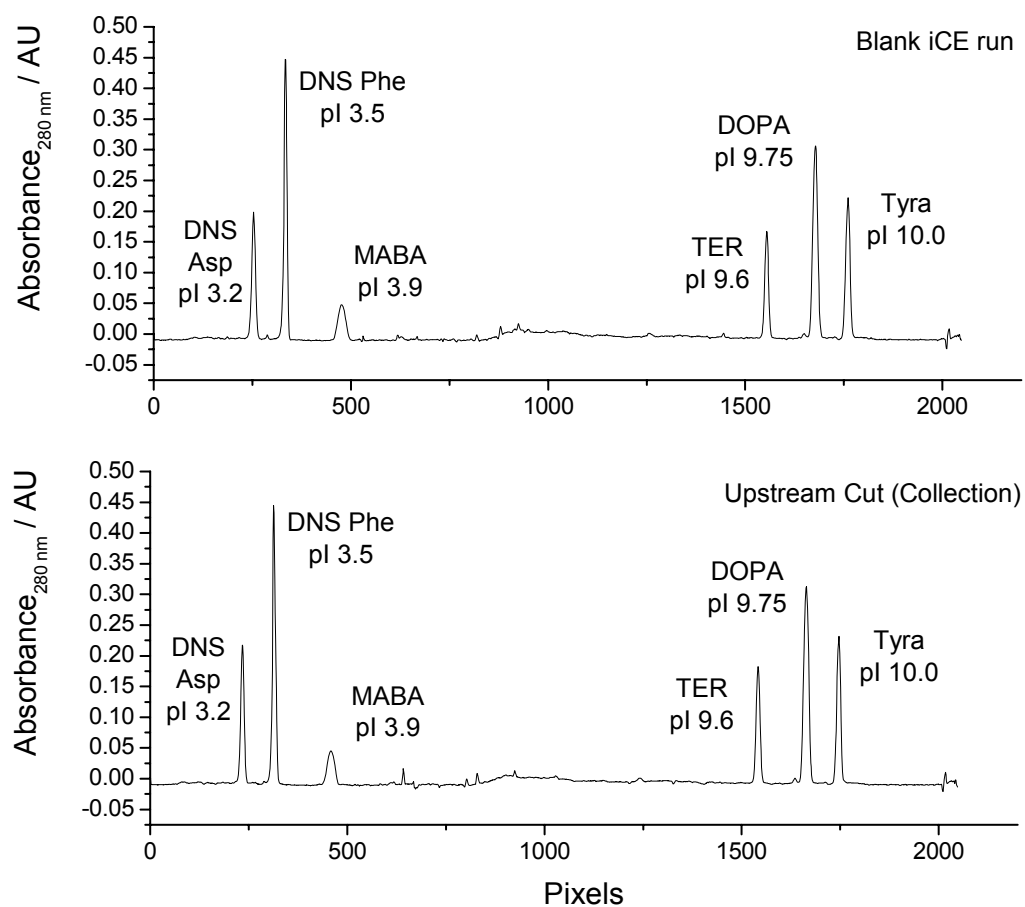


Figure 32. ICIEF traces of the UV-transparent carrier ampholytes (blank sample) and the upstream cut (feed)

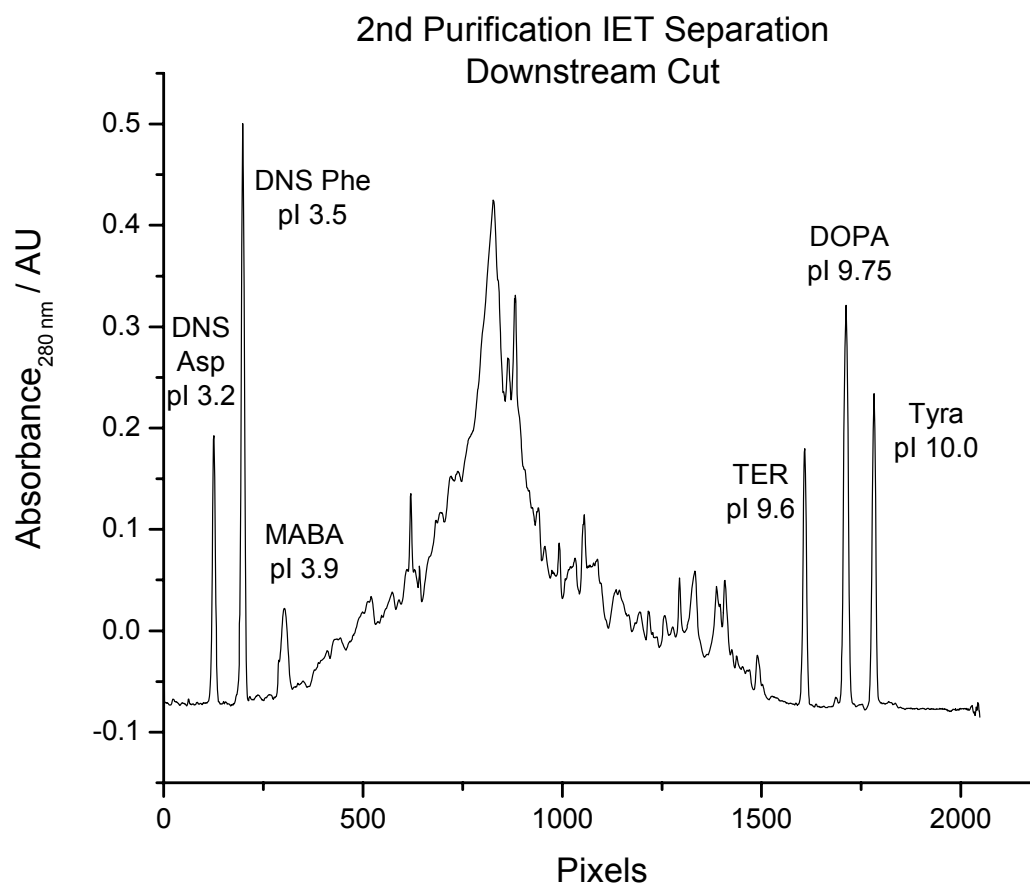


Figure 33. ICIEF traces of the downstream sample where UVCAs were collected

characterized by ^1H NMR and the final products were characterized by ICIEF using the full-column imaging iCE280 instrument.

The UVCAs (2 equiv itaconic acid + 6 equiv acrylic acid) was successfully desalted and purified in two IET steps by first removing the salts and separating the unreacted acrylic acid and/or other weak acids from the semi-purified UVCAs by feeding the sample in the downstream and collecting it in the upstream. Next, the collected UVCAs were fed in the upstream and the purified material was collected in the downstream.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Binary IET Separations and the pH Gradients

Pharmalytes are known to produce a relatively linear pH gradient. However, by adding a significant amount of UV-absorbing PEHA carrier ampholytes to the mixture, the pH gradient is distorted in the capillary of the iCE280 instrument. Therefore, to compensate for this distortion, two gradients must be calculated on the opposite sides of the concentration boundaries of the PEHA CAs. By choosing 2 – 3 pI markers on both sides of the concentration boundaries of the UV-absorbing PEHA carrier ampholytes, the correct local pH gradient can be determined. Similarly, by choosing pI markers within the range of the UVCAs, the pH gradient of the synthesized carrier ampholytes can be determined.

4.2 Blending UVCAs

To obtain a smooth absorbance distribution for the mixture of the UV-absorbing carrier ampholytes, different products from the various Michael additions were mixed. By mixing a sufficient amount of each product (1 – 8 equiv acrylic acid + 1 – 2 equiv itaconic acid / 7 – 6 equiv acrylic acid), the amount of UVCAs in the low pI range became low (Figure 34) because relative to the higher pI range

ampholytes, there was not enough material to produce a sufficient absorbance signal. Therefore, an appropriate amount of the UV-absorbing PEHA carrier ampholytes from 1, 7, 8 equivalents of acrylic acid and 2 equivalents of itaconic acid + 6 equivalents of acrylic acid, were blended together. The electropherogram from the ICIEF is shown in Figure 35.

Here, the UVCAAs are distributed somewhat unevenly, with two major breaks in the pH range 3 – 10. Again, the low pI range contains a much smaller amount of UV-active ampholytes than the higher pI range. Therefore, to mitigate this problem, a sample of 8 equivalents of acrylic acid UV-absorbing PEHA carrier ampholytes was desalted, purified, and the lower pI range was concentrated by running a binary IET separation on the modified BF200IET unit.

The analyte was loaded downstream and the higher pI ampholytes were allowed to leave the downstream compartment. The current, potential, power, pH and conductivities in all compartments, were monitored. Once the pH went down in the downstream and the conductivity of the upstream increased, IET was stopped. By mixing the downstream (feed) sample with the previously blended

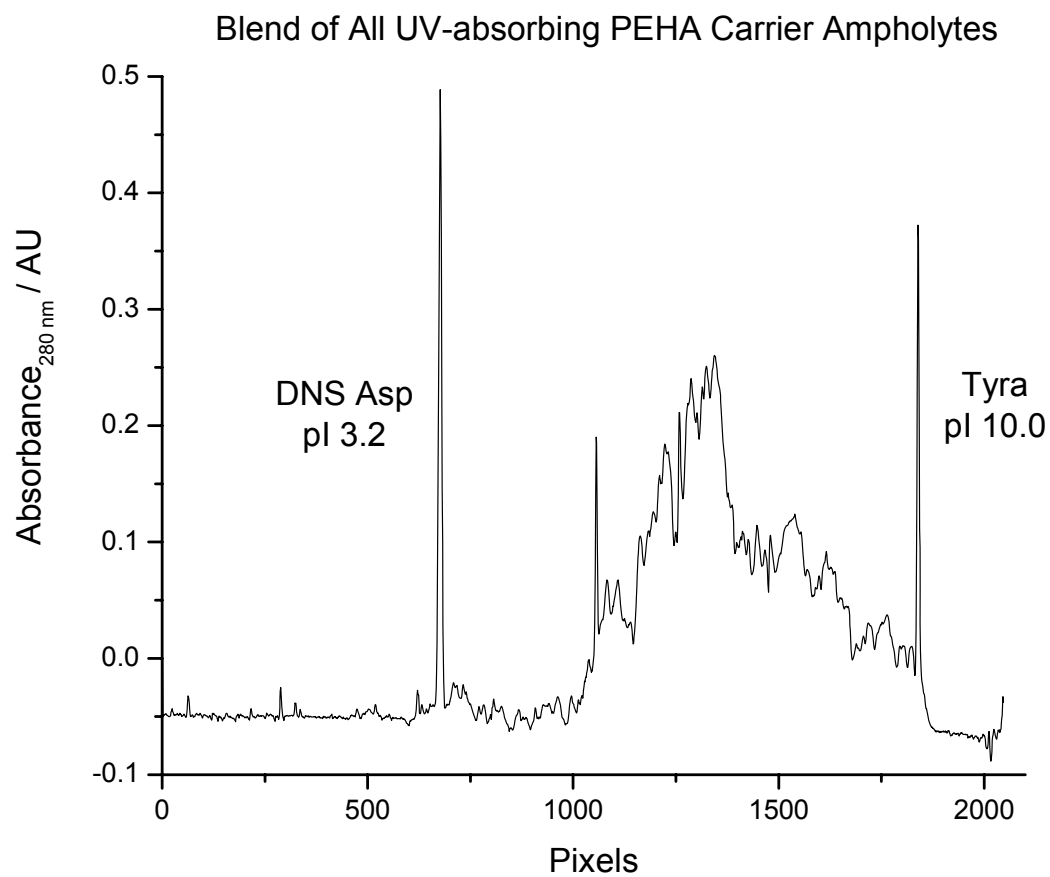


Figure 34. ICIEF trace of blend of all UVCAAs (1 – 8 equiv acrylic acid + 1 – 2 itaconic acid / 7 – 6 equiv acrylic acid)

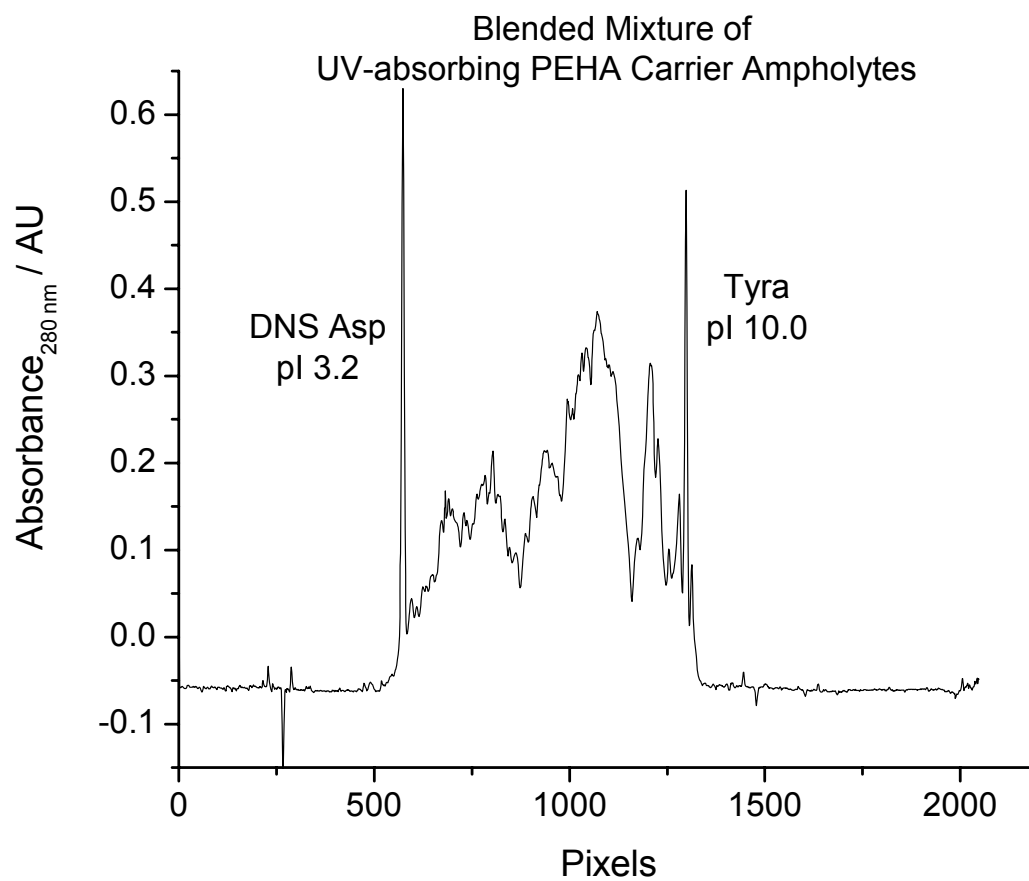


Figure 35. ICIEF trace of blend of selected UVCAAs (1, 7, 8 equiv acrylic acid and 2 equiv of itaconic acid + 6 equiv acrylic acid)

mixture of UV-absorbing carrier ampholytes, the ICIEF trace was greatly improved as shown in Figure 36. The major breaks were filled in and the signal in the lower pI range was greatly enhanced. Figure 37 shows the final blend of UVCAs, which had the mole ratio of the UV-absorbing chromophore of 1 : 0.1 : 0.6 : 0.8 for the 1 equiv : 7 equiv : 8 equiv : 2 equiv itaconic acid + 6 equiv acrylic acid.

4.3 Determining the Operational pI Value of a Buffering Membrane

A polyacrylamide-based isoelectric membrane (pI 4.4) was used to separate the blended mixture of PEHA-based UVCAs in the BF200IET unit. After the IET was complete, the samples were analyzed on the iCE280 instrument. The electropherograms for the downstream and upstream samples are shown in Figure 38. The pI markers chosen were: DNS Asp (pI 3.2), DNS Phe (pI 3.5), TER (pI 9.6), and TYRA (pI 10). The pH gradients for the UV-absorbing PEHA carrier ampholytes were calculated. Figure 39 shows a pI vs pixel number calibration plot for upstream and downstream cuts.

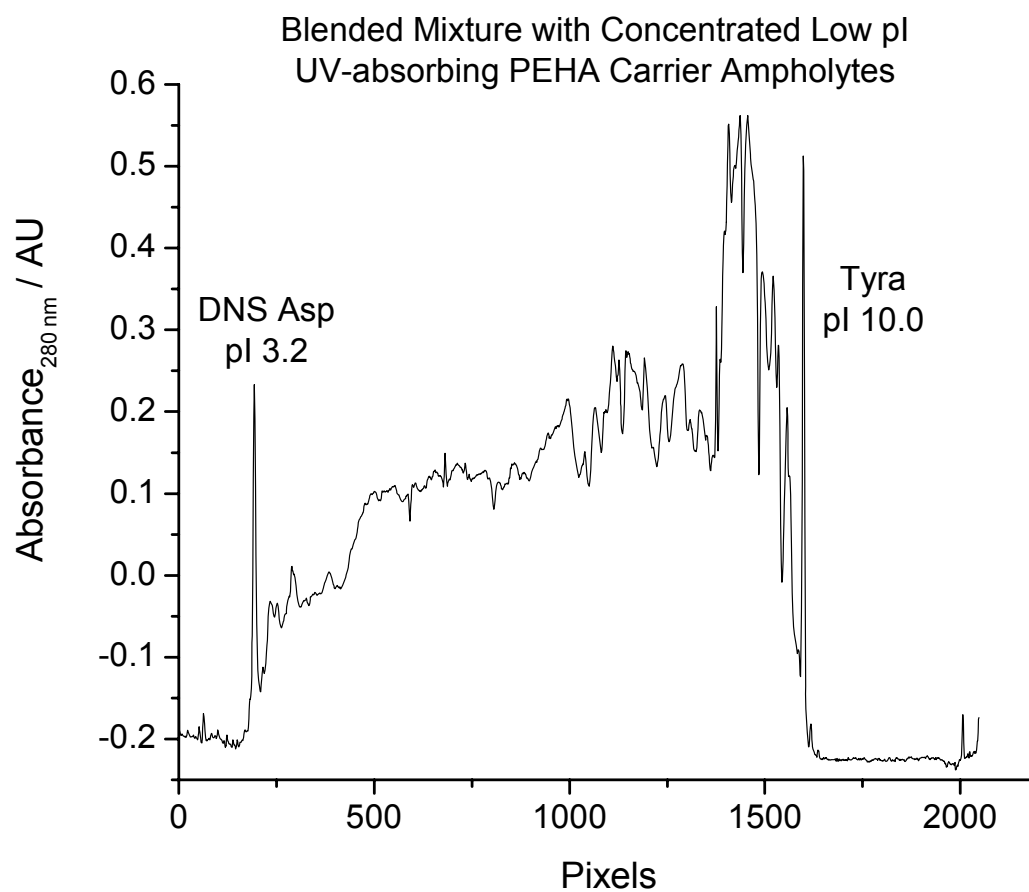


Figure 36. ICIEF trace of an improved blend of selected UVCAs and concentrated low pI UVCAs

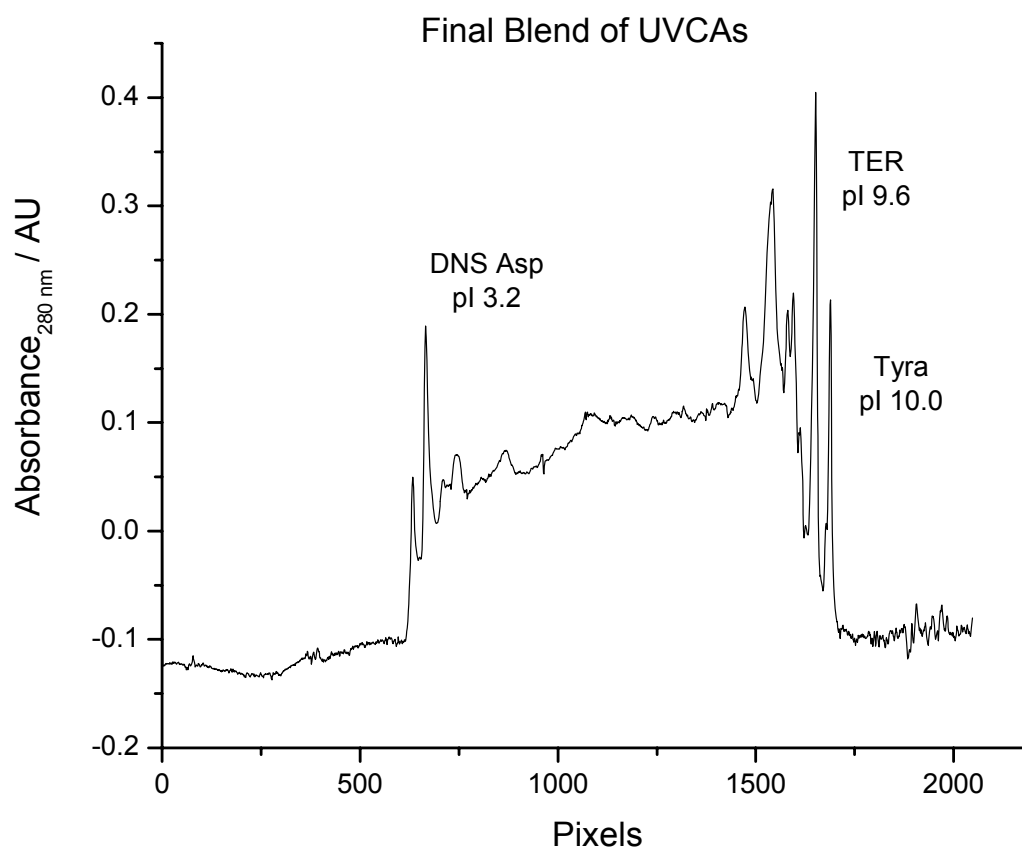


Figure 37. ICIEF trace of the final blend of UVCAs

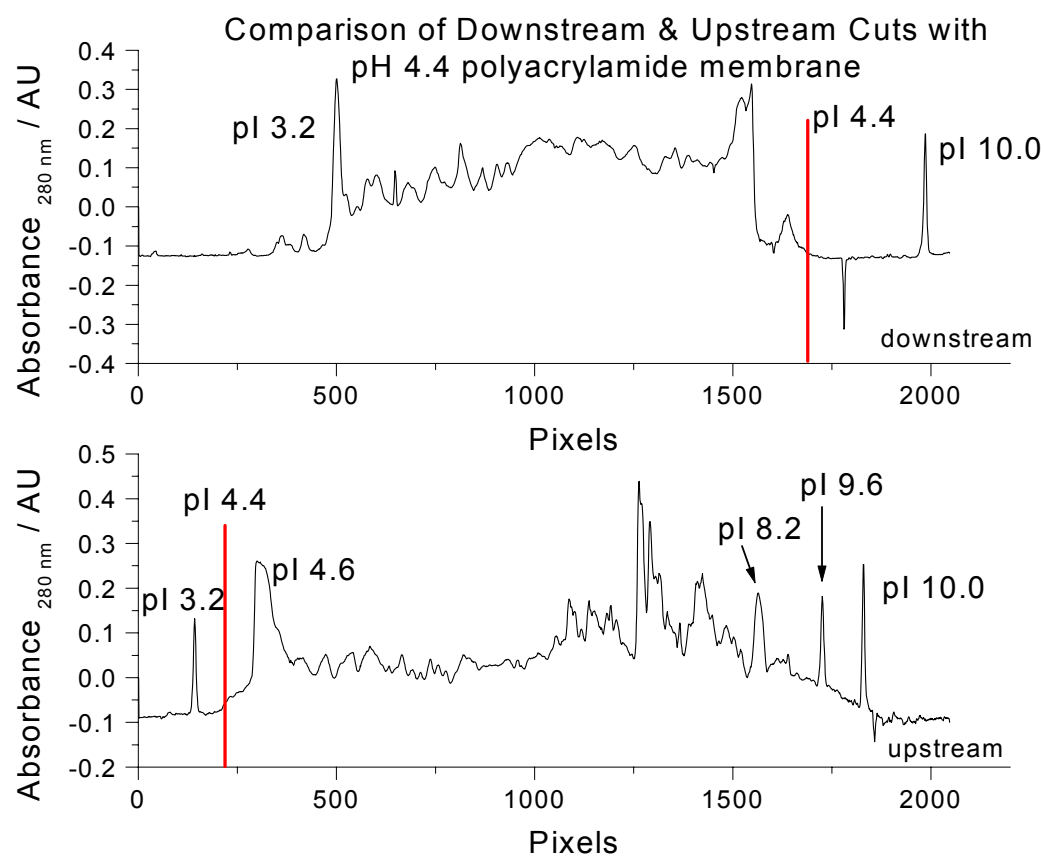


Figure 38. ICIEF traces comparing the downstream and upstream sample cuts using pI 4.4 polyacrylamide-based membrane

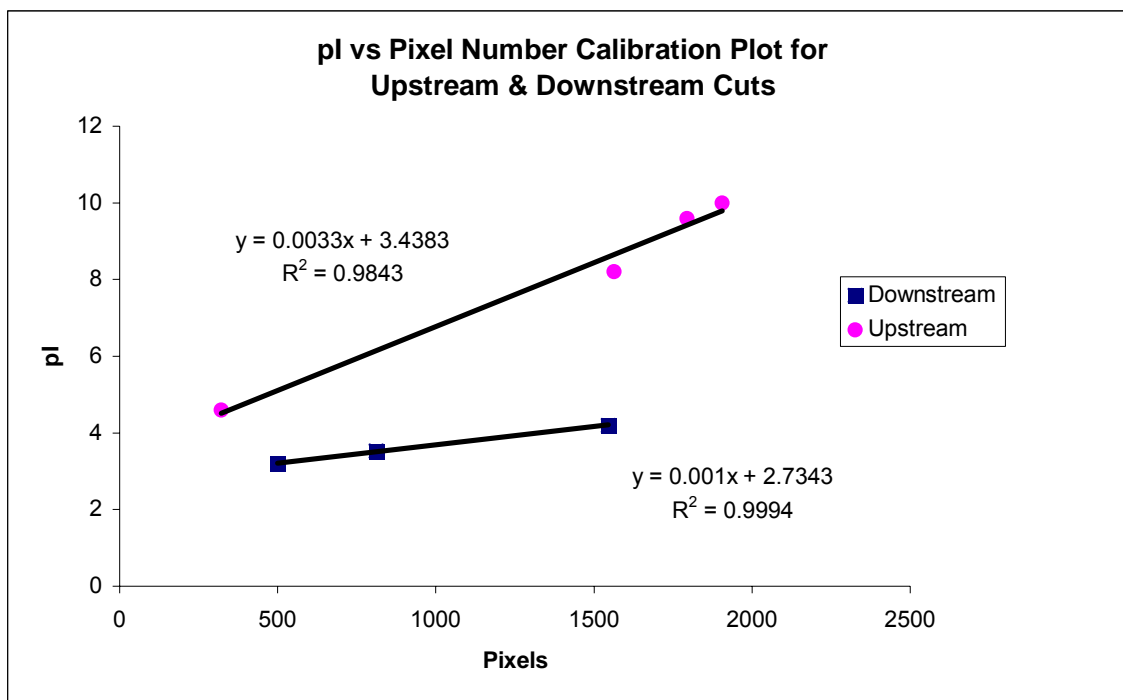


Figure 39. pl vs pixel number calibration plot for upstream and downstream cuts

4.4 Summary

A sufficient amount of the UV-absorbing PEHA carrier ampholytes from 1, 7, 8 equivalents of acrylic acid and 2 equivalents of itaconic acid + 6 equivalents of acrylic acid, were blended together. This mixture was combined with another batch of the 8 equivalents acrylic acid-containing UV-absorbing PEHA carrier ampholytes, in which a sufficient amount of the higher pI ampholytes were migrated out of the feed chamber, effectively concentrating the lower pI ampholytes. This mixture produced the most suitable ICIEF electropherogram. These blended UV-absorbing PEHA carrier ampholytes were used to test the operational pI value of a nominal $pI = 4.4$ polyacrylamide-based isoelectric membrane. According to the ICIEF results of the downstream and upstream samples, different pH gradients were evident in the UV-transparent carrier ampholyte-filled (Pharmalytes) and the UV-absorbing carrier ampholytes (PEHA-based).

CHAPTER V

CONCLUSIONS

PEHA-based UVCAs were successfully synthesized in approximately the pH range of 3 – 10. The UVCAs were synthesized by first attaching a chromophore, PhOPrBr, to the PEHA backbone, which was verified by ^1H NMR. In a Michael addition, this intermediate was subsequently reacted with increasing numbers of equivalents of acrylic acid. To improve the component distribution in the UVCA mixture, itaconic acid was also added. These reactions were also verified by ^1H NMR and ICIEF.

Using PEHA as the carrier ampholyte backbone has the main advantage of providing a wide pKa range. By controlling the amounts of acrylic acid added, the pH range of UVCAs produced varies across the pH range. The fractions were blended to produce a UVCA mixture that had an even UV absorbance profile across the entire pH gradient. Furthermore, attaching itaconic acid to the UVCA mixture filled the areas in which there were still gaps, mainly near pH 6.7. Once an optimal mixture of UVCAs was blended, an IET separation was done and results from the ICIEF have shown that a cut was made at approximately pH 4.4, using a polyacrylamide isoelectric membrane of nominal pH 4.4. The pH gradients for the UVCA mixture were calculated for each subfraction of the IET

separation. Full-column imaging CIEF proved to be invaluable in characterizing the UVCA mixtures and the results of the IET separations.

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VITA

Ann Hwang received her B.S. degree in chemistry from the University of Illinois at Urbana – Champaign in 2000 and her M.S. in chemistry from Texas A&M University in 2005. While at the University of Illinois, she worked under the direction of Professor Kenneth L. Rinehart in isolating bioactive materials from a fungus. She has also worked at the Naval Research Laboratory in Washington, D.C. under the guidance of Dr. Andrew Purdy synthesizing high dielectric polymers for capacitors and large cubic gallium nitride crystals for laser applications. She can be reached at: 702 Gaytha Circle #11, Bryan, TX 77801.